CHAPTER SIX:
MOLECULAR CHARACTERIZATION OF THE *gol* ALLELE OF *Brca1*

6.1 INTRODUCTION

The two *Brca1* deletion alleles generated in this study, *gol* and *Brca1*$^{-}$, are phenotypically different despite their structural similarity. Data presented in previous chapters demonstrated that neither *Brca1*$^{-/-}$ ES cells nor mice could be generated, but that *gol/gol* ES cells are viable and grow normally, although they are hypersensitive to DNA damage. The Brca1^{gol} protein is able to localize to the nucleus and is detected in nuclear foci both before and after DNA damage.

The final goal of this project was to investigate the consequences of the *gol* and *Brca1*$^{-}$ mutations on the regulation of Brca1, with a view toward explaining the difference in viability between the two alleles. Both were designed to replace exon 2 with selection cassettes transcribed in the opposite direction from the transcriptional orientation of *Brca1*. Aside from carrying different selection markers, the two alleles differ only in the amount of intronic sequence they delete: ~2 kb more genomic sequence is deleted in the *gol* allele (Figure 6.1).

6.1.1 Role of the PGK promoter

Initially it was hypothesized that the *gol* allele gave rise to an alternative *Brca1* transcript, driven by the PGK promoter from the *Puro* selection cassette. This promoter is reversed in relation to the direction of *Brca1* transcription, but previous reports have suggested that the PGK promoter may be able to act bidirectionally (Johnson and Friedmann, 1990; Abeliovich, 1992; Scacheri, 2001). As exon 2 of *Brca1* contains the translational start site, translation of an alternative transcript is likely to initiate from a downstream, in-frame AUG codon, of which there are three near the 5' end of *Brca1*. Experiments described in this chapter indicate that while the PGK promoter does appear to be able to act bidirectionally in ES cells, it does not appear to be affecting transcription of *Brca1* from the *gol* allele. Furthermore, while different isoforms of *Brca1* are expressed from the *gol* allele (the most prominent being the *Brca1 ΔX.2* isoform, described in Chapter 3, in which exon 1 is spliced

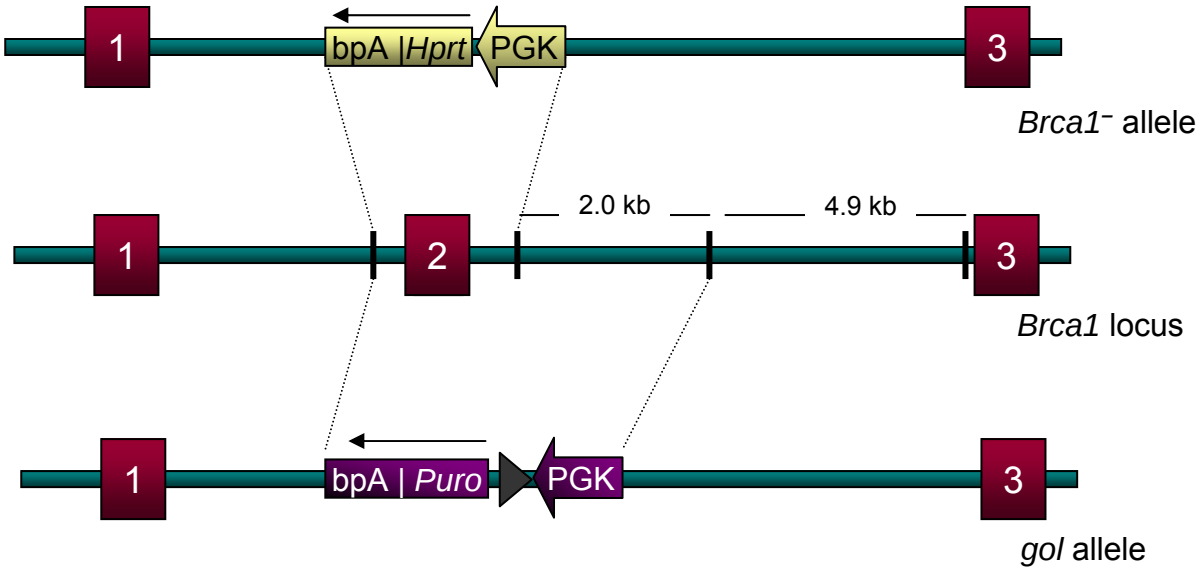


Figure 6.1: The *Brca1*⁻ and *gol* alleles (5' end).
The *Brca1*⁻ and *gol* alleles have two major differences: their selection cassettes, and the amount of intronic sequence they replace. ~2 kb more intronic sequence is deleted in the *gol* allele.
The grey triangle represents a *loxP* site.
Not drawn to scale.

directly to exon 3), no transcripts unique to the *gol* allele were detected by several different assays. This suggests that if a non-endogenous promoter is involved in the transcription of *Brca1*, the level of the novel transcript is too low to be detected by the methods used in this study.

6.1.2 Transcriptional control of the *Brca1*$^{-}$ and *gol* alleles

Although *Brca1* mRNA does not seem to be overexpressed in cells carrying a *gol* allele, the ratio of mutant-to-wildtype *Brca1* transcripts differs between *+/gol* and *+/–* ES cells, and Brca1 protein is more abundant in cells carrying a *gol* allele. This increase in protein level (or stability) supports the idea that translation of Brca1 initiates at a downstream AUG codon. The lack of a noticeable change in the *Brca1* mRNA level in cells carrying a *gol* allele may be due to overall regulation of *Brca1* levels in the cell. While there would then be no difference in the type of transcript (*Brca1 ΔX.2*) transcribed from *gol* or *Brca1*$^{-}$, the inviability of *–/–* ES cells and embryos (and viability of *gol/gol* ES cells) generated in this study suggests that the relative amount of transcription from *gol* is higher than from *Brca1*$^{-}$. It is hypothesized that the extra 2 kb of intronic sequence deleted in the *gol* allele may carry a transcriptional suppressor. An alternative hypothesis is that the *Puro* selection cassette carried by the *gol* allele serves as a transcriptional enhancer.

6.1.3 The interaction of Brca1^{gol} and Bard1

Bard1 is a RING-domain binding partner of Brca1 (Wu, 1996). As discussed earlier, the heterodimerization of Brca1 and Bard1 is thought to be of major importance in nuclear import, nuclear retention, and functionality of Brca1 (Fabbro, 2002). The interaction of Bard1 and Brca1^{gol} was assessed by co-immunoprecipitation. Bard1 appears to have a reduced amount of interaction with Brca1^{gol}, which extends the current knowledge about the dependence of Brca1 on its interaction with Bard1 for functionality. Furthermore, this result supports the supposition that the Brca1^{gol} protein does not have a fully-functional RING domain, as is predicted based on sequence and structure analyses of the *gol* allele.

6.2 RESULTS

6.2.1 Brca1^{gol}: predicted translation initiation sites

Exon 2 of *Brca1* encodes both the translational start site and part of the highly-conserved N-terminal RING domain. This domain is known to be important for protein-protein interactions, and is where Brca1 interacts with Bard1, its binding partner and putative nuclear chaperone (Wu, 1996). A look at the protein structure of the entwined RING domains of human BRCA1 and BARD1 (Figure 6.2a) shows that the region encoded by exon 2 (Figure 6.2b and c) is an integral part of the interaction structure. Indeed, *in vitro* studies have established that some point mutations in the RING domain of BRCA1 abolish its ability to heterodimerize with BARD1 (Wu, 1996; Brzovic, 1998; Brzovic, 2001a; Joukov, 2001b; Morris, 2002).

The viability of *gol/gol* ES cells suggests that the *gol* allele gives rise to a functional protein. As the translational initiation site of *Brca1* has been deleted in the *gol* transcript, and no AUG codons are present in exon 1, translation of this protein may initiate from an in-frame, downstream AUG codon. Three such AUG codons exist near the 5' end of *Brca1*, in exons 5 and 6 (Figure 6.3).

A favored model of eukaryotic translational initiation is the scanning model, whereby a ribosome binds to the 5' end of a message and scans in a 5'–3' direction until an AUG codon is encountered (Kozak, 1978; Kozak, 1989; Kozak, 1997). However, the first in-frame AUG encountered downstream from the exon 2 deletion is situated just upstream of the NES described in section 1.7, meaning that the Brca1^{gol} protein would lack the RING domain but have both an NES and NLSs. Experiments reported by Fabbro *et al.* using transiently expressed BRCA1 and BARD1 proteins suggest that a BRCA1 protein which lacks the RING domain but possesses a functional NES will be located mainly in the cytoplasm (Fabbro, 2002). This directly contradicts experimental results reported in Chapter 5 which showed that the localization of Brca1 in *gol/gol* and wildtype cells is very similar (Figure 5.9). Additionally,

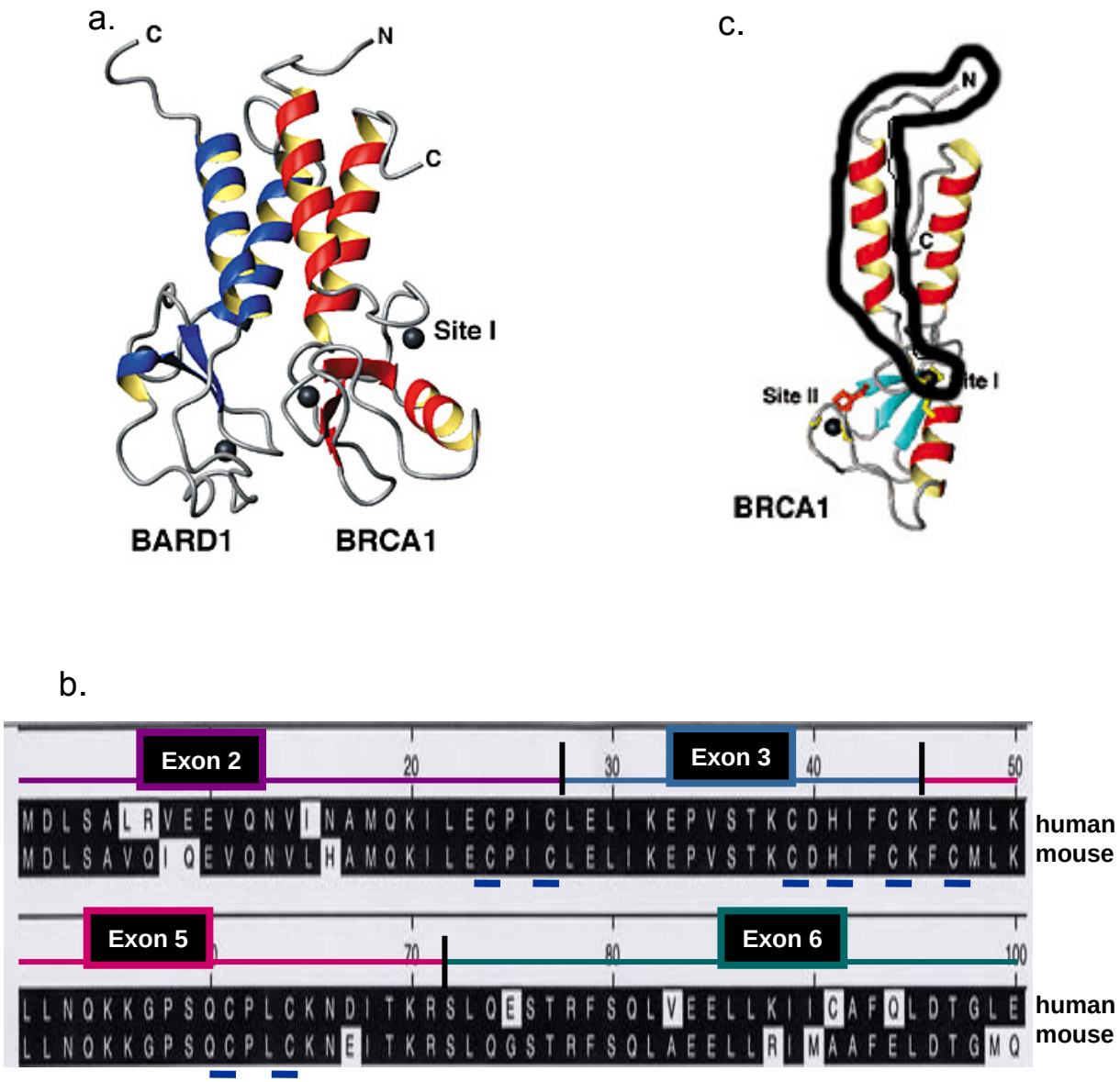
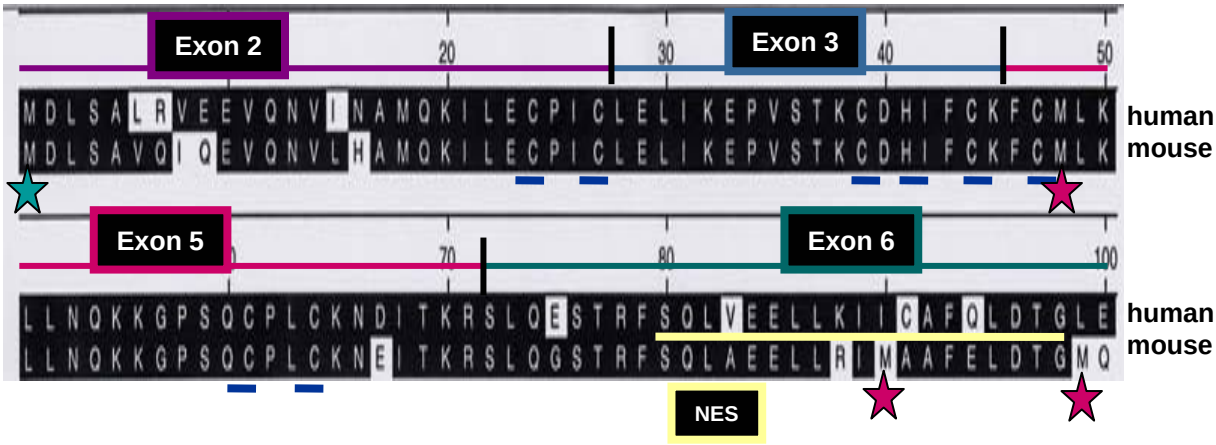


Figure 6.2: Structure of the BRCA1/BARD1 RING domain interaction.
a. Structure of the human BRCA1/BARD1 RING-RING interaction structure. **b.** Mouse-human Brca1 protein alignment including the first 100 residues of Brca1. Black highlighting indicates identical residues. Exon regions are indicated and the key cysteine (C) and histidine (H) residues of the Zn-finger RING motif are underlined in blue. **c.** The region encoded by exon 2 is circled on the BRCA1 structure.
Structural diagrams taken from (Brzovic, 2001).

a.



b.

Kozak:	GCC	PuCC	AUG	G
exon 2:	GAA	GAA	AUG	GAU
exon 5:	UUU	UGU	AUG	CUG
exon 6:	GAA	AUA	AUG	GCU
exon 6:	ACG	GGA	AUG	CAG

Figure 6.3: In-frame, 5' AUG codons of *Brca1*. a. Human-mouse Brca1 RING domain alignment, as in Figure 6.5. Red stars indicate potential downstream translational start sites, a green star denotes the normal start site. Exons and the nuclear export signal (NES) are indicated. **b.** Contextual setting of the endogenous and downstream AUG codons compared to the ideal Kozak consensus sequence. Bases in the +4 and -3 positions considered ideal in the Kozak model are underlined.

many experiments by Marilyn Kozak demonstrate that translational efficiency is highly influenced by the context of the AUG start codon, especially by the bases in the -3 and +4 positions (relative to the A of AUG) (Kozak, 1981). The ideal configuration, as determined both by experimentation and cataloguing of archived sequences, is a purine at -3 and a guanine (G) at +4 (Kozak, 1981; Kozak, 1984; Kozak, 2001). Similar experiments have suggested that if the first AUG encountered by a ribosome is in a poor context, it may not be recognized as a start site, and the ribosome will continue scanning (Kozak, 1978; Kozak, 1986).

Figure 6.3b shows the contextual setting of the downstream AUG codons of *Brca1*. The first AUG, in exon 5, is in a very poor context and *in vitro* experiments done by Kozak strongly suggest that it would be used rarely, if at all, as a start codon (Kozak, 1997). The second AUG codon, in exon 6, has a much more favorable context. Furthermore, this second AUG is located in the middle of the NES. If translation began from this site, the resulting Brca1^{gol} protein would lack both the RING domain and the NES, and, according to data reported by Fabbro *et al.*, would be able to localize to the nucleus using the NLSs coded by exon 11 (Fabbro, 2002). The use of the second AUG codon would correlate well with the immunolocalization data presented in Chapter 5. It is possible that translation initiates from more than one start site, possibly generating two forms of the protein with different cellular localizations. There is additionally the possibility that alternative start codons (codons which differ from AUG by one base, such as ACG or CUG) may be used for initiation (Touriol, 2003). The use of alternative start codons is not common, and several studies have indicated that the context of these sites is very important in determining how effectively they work (Mehdi, 1990; Boeck and Kolakofsky, 1994). Analysis of the *Brca1* sequence indicates that none of the first in-frame occurrences of alternative start codons occurs in a good context, suggesting that if they are used, the protein translated from them would probably be expressed at a low level. In this study, no experiments were performed to determine the start codon used in Brca1^{gol}.

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While downstream translational initiation of Brca1^{gol} seems likely – not least because *gol/gol* ES cells are viable – downstream initiation might be equally likely for transcripts of the *Brca1*$^{-}$ allele. That these two alleles are not phenotypically identical suggests that either translation from a downstream AUG in the *Brca1*$^{-}$ transcript is less robust, or that the *gol* allele produces a different transcript than does the *Brca1*$^{-}$ allele. In the case of the *gol* allele, this hypothetical different transcript could be expressed under the control of the PGK promoter from the *Puro* selection cassette, reversed in relation to the direction of *Brca1* transcription (see Figure 6.1). A reversed PGK promoter has been shown to drive expression of a reporter gene *in vitro* (albeit at low efficiency), and a few instances of aberrant transcription arising from a reversed PGK promoter have been reported in the literature (Johnson and Friedmann, 1990; Abeliovich, 1992; Scacheri, 2001).

Brca1^{-} also carries a reversed PGK promoter as part of its *Hprt* selection cassette (see Figure 6.1). The difference between these two promoters is their proximity to exon 3 – the *Puro* selection cassette is ~2 kb closer to exon 3 than is the *Hprt* cassette (Figure 6.1). Based on evidence described in Chapters 3 and 4, as well as data reported by Ludwig *et al.* (from whom the derivative of the *Brca1*-Hprt-TV was obtained), the *Brca1*$^{-}$ allele behaves as a null allele – that is, homozygous mutant embryos or ES cells are not viable (Hakem, 1996; Liu, 1996; Ludwig, 1997). This suggests that the level of transcription of the *Brca1*$^{-}$ allele driven by the reversed PGK promoter is negligible at best. If the position of the reversed PGK promoter is important for expression of *gol*, this hints that intronic sequence near the PGK promoter in the *gol* allele may be important for expression, that perhaps the promoter is using nearby intronic sequence as an alternative transcriptional start site.

6.2.2 PGK is a bidirectional promoter in ES cells

Some evidence generated from *in vivo* expression and *in vitro* reporter-gene experiments suggest that the bidirectional activity of PGK may be cell-, tissue-, or gene-specific (Johnson and Friedmann, 1990; Abeliovich, 1992). To test if the reversed PGK promoter is effective in ES cells, three *Puro*

vectors (*Puro* expressed using either the PGK promoter, a reversed PGK promoter, or no promoter) were electroporated into wildtype ES cells which were then selected in puro-containing medium. Table 6.1 shows that the reversed PGK promoter does appear to be able to drive expression of a reporter gene in ES cells, albeit at a lower level than the control promoter (~15%). This compared favorably with a previously-reported *in vitro* study, in which a reporter gene driven by the reversed PGK promoter was expressed at ~10% of the normal level (Johnson and Friedmann, 1990).

As electroporation generally results in tandem integration, puro-resistant colonies could result from integration of the revPGK-*Puro* cassette if read-through occurred between adjacent copies. However, while a tail-to-tail integration would provide such a read-through substrate, the PGK promoter would have to traverse two ampicillin-resistance cassettes to reach the reporter gene, making this an unlikely event (Figure 6.4).

While this experiment indicated that the PGK promoter appears to be able to act bidirectionally in ES cells, an added layer of complexity is present in the *gol* and *Brca1*$^{-}$ alleles, as the promoter is also driving transcription of a gene in the forward direction. Only one *in vitro* dual-reporter gene experiment has been reported in the literature; it suggested the possibility of decreased expression of the reporter construct in the reverse direction when a second gene was being transcribed in the forward direction (Johnson and Friedmann, 1990). However, if the PGK promoter proves instrumental in expression of *gol*, this question can be directly investigated *in vivo* using the *c2* and *gol* alleles; the *c2* allele has not yet undergone Cre-mediated recombination, so the reversed PGK promoter is not actively transcribing *Puro* (although this does not mean that it is not operational), while in the *gol* allele, *Puro* is actively being transcribed (compare Figure 3.3b and c). Of the few groups which have reported aberrant expression from a reversed PGK promoter, only two determined the sequence of the resulting transcript. Both groups found that part of the reversed promoter itself was included in the resulting transcript: 308 bp of promoter sequence was included in one case, while in the second study, which involved multiple transgenic lines, the amount of

Table 6.1: Efficiency of the reversed PGK promoter in ES cells.
Total puro-resistant colonies resulting from electroporation of plasmids carrying *Puro* driven by the PGK promoter (PGK), reversed PGK promoter (revPGK), or no promoter into wildtype ES cells.

Vector	Colonies experiment 1	Colonies experiment 2	Percentage of control experiment 1	Percentage of control experiment 2
PGK-*Puro*-bpA	1880	2592	100	100
revPGK-*Puro*-bpA	256	448	14	17
Puro-bpA	0	0	0	0

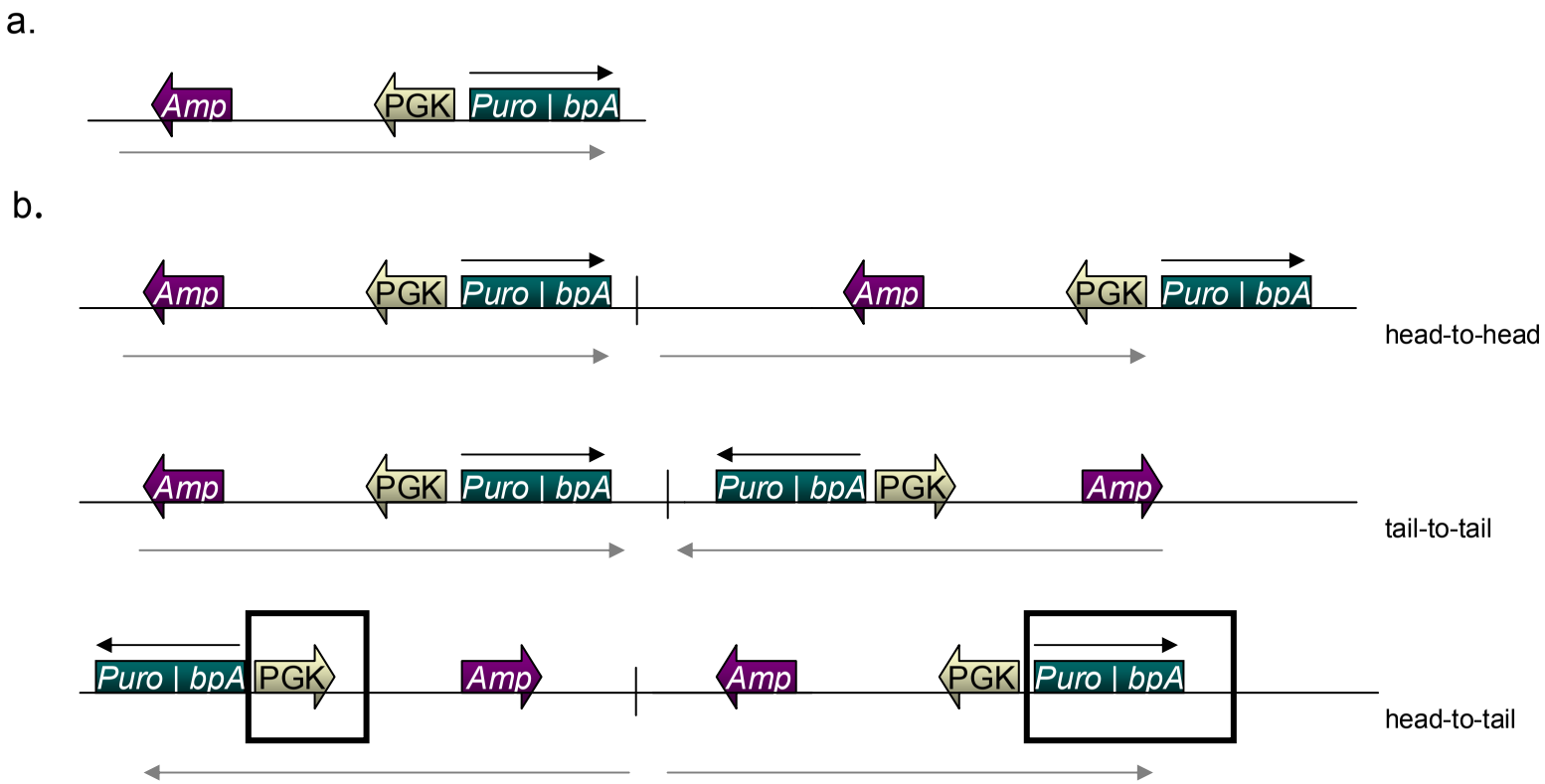


Figure 6.4: Possible orientation of tandem integrations of the revPGK-*Puro*-bpA plasmid.
a. The revPGK-*Puro*-bpA construct, linearized at the 3' end of the *Puro* gene. **b.** The three possible orientations of multiple insertions of the revPGK-*Puro*-bpA construct. Only a tail-to-tail integration forms a potential "read-through" PGK-*Puro*-bpA cassette (boxed), and two ampicillin-resistance (*Amp)* cassettes lie between promoter and coding region, making this an unlikely event.
Total vector size is 4.5 kb; the revPGK-*Puro*-bpA cassette is 1.5 kb. Figure is not drawn to scale.

promoter sequence varied (Abeliovich, 1992; Scacheri, 2001; Scacheri, 2003).

6.2.3 Multiple products are detected in a 5' RACE assay using *gol/gol* ES cell RNA

To try and identify transcripts expressed from the reversed PGK promoter, 5' Rapid Amplification of cDNA Ends (5' RACE), which utilizes known sequence from a transcript to amplify an unknown 5' region, was performed using total RNA from *gol/gol*, *+/–*, and wildtype ES cells. This analysis was expected to indicate the presence of an alternative transcript, lacking exon 1, present in *gol*-expressing cells but not in wildtype cells. Any putative alternative transcript might or might not be present in *+/–* cells, depending on the activity of the reversed PGK promoter in the *Brca1⁻* allele. 5' RACE was also expected to reveal if any intronic sequence was present in the putative PGK-driven transcript. Figure 6.5a shows that the predominant product amplified from *+/–* cells is a wildtype *Brca1* product. Two alternative products are also generated: the *ΔX.2 Brca1* isoform described in Chapter 3, in which exon 1 is spliced to exon 3, and an additional smaller product. *gol/gol* samples also show this smaller product, but their most abundant product is the *Brca1 ΔX.2* isoform – and no wildtype product is observed. A comparison of 5' RACE products from wildtype and *+/–* cells (Figure 6.5b) demonstrates that the wildtype *Brca1* allele also produces the smaller alternative product.

Sequence analysis of this smaller product indicates that it is a mix of two products, which end at the two AUG codons of exon 6 (Figure 6.5c). Random subcloning and sequencing of many 5' RACE products from *gol/gol* cells indicate that while other products are produced (including one which ends at the exon 5 AUG), the exon 6-AUG and *Brca1 ΔX.2* products are by far the most common. Analysis of a large number of subcloned *gol/gol* 5' RACE products did not identify any product containing sequence from either the PGK promoter or a *Brca1* intron (except for an intron 9-containing product which was also detected in wildtype samples; data not shown).

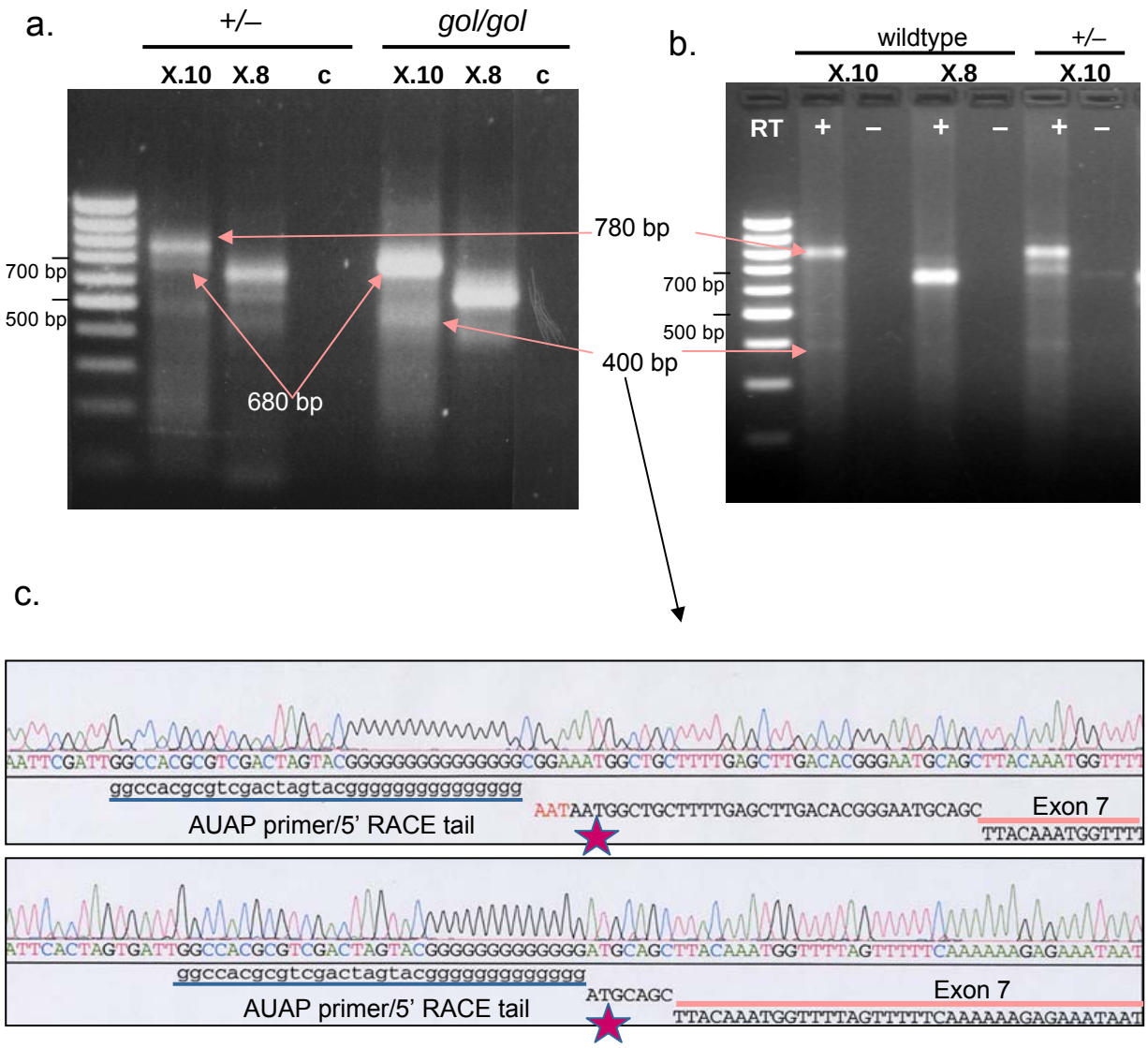


Figure 6.5: 5'RACE analysis of wildtype, +/–, and *gol/gol* ES cell RNA. **a.** 5' RACE of RNA from ES cells of indicated genotypes, followed by PCR with AAP and *Brca1* X.10R or *Brca1* X.8R2 primers. A variety of products are amplified from *gol/gol* and +/– samples. No unique products are observed in *gol/gol* samples. The 780 bp product is wildtype *Brca1*. **b.** 5' RACE followed by PCR (as in a.) using wildtype and +/- RNA samples. The smaller, 400 bp product also appears in wildtype *Brca1* X.10–amplified samples. **c.** Sequence analysis of the 400 bp band. A mix of two products is observed, each ending at one of the two exon 6 AUGs (red stars). "RT +" or "RT –" indicates if reverse transcriptase was added to the 5' RACE reaction or not. "c"=non-primer control PCR reaction, X=exon, AUAP=5'RACE tail sequence, AAP=Abridged Anchor Primer.

While 5' RACE did not reveal the presence of unique *Brca1* transcripts in *gol/gol* cells, the possibilities remained that such transcripts are expressed at a low level and were not detected, or that the alternative transcript starts further 3' than the exon 10 primer used for the 5' RACE reaction (see section 2.4.3 for sequence of the primer), and thus would not be detected by this experiment.

6.2.4 Detection of PGK sequences by Northern blot analysis

As two previous reports of aberrant transcripts expressed from a reversed PGK promoter have demonstrated that sequence from the promoter itself was included in the transcripts (Johnson and Friedmann, 1990; Abeliovich, 1992; Scacheri, 2001), potential inclusion of PGK promoter sequence in the *gol* transcript was tested by Northern blot.

A Northern blot of total RNA from wildtype and *gol/gol* ES cells was probed with a DNA probe comprising both strands of sequence from the first half of the PGK promoter (5'  3' – dark half). No transcripts were identified in any sample using this probe (data not shown). Hybridization and washing of this blot were done in tandem with a second Northern blot; successful hybridization of the second blot suggested that faulty technique did not account for the lack of signal. The blot used was subsequently and successfully hybridized with a control probe. RT-PCR analysis was also used to try to identify a potential PGK–*Brca1* fusion, using primers in the reversed PGK promoter paired with downstream *Brca1* reverse primers, but no such fusion transcript was identified (data not shown).

6.2.5 Direct sequencing of 5' RACE products

While no evidence for the use of a reversed PGK promoter was identified from subcloning and sequencing 5' RACE products, and the inclusion of the PGK promoter in a *gol* transcript is not supported by Northern blot or RT-PCR analyses, it was still possible that transcription is driven by the reversed PGK promoter but promoter sequence is not included in the resulting transcript.

Therefore, an attempt was made to identify a novel transcript driven by the PGK promoter by directly sequencing a pool of PCR-amplified 5' RACE products. By sequencing the entire pool of products, the presence of transcripts with novel 5' sequence should be indicated by an "interference pattern" in the sequence where the difference occurs. As a control, a pool of products generated using +/– RNA was used; a proportion of these transcripts lack exon 2, so a difference is expected at the exon 2–exon 3 boundary (inset, Figure 6.6). *gol/gol* and +/– RNA was subjected to 5' RACE and sequenced using several reverse *Brca1* primers.

As expected, sequence of the +/– 5' RACE product pool shows an "interference pattern" at exon 2 (Figure 6.6). No such pattern is seen in *gol/gol* cells at any point between exons 1 and 9 (Figure 6.7 shows two representative sequence trace files). A second 5' RACE reaction was run, but the results did not differ. It should be noted that *Brca1* does not have an exon 4, for historical reasons (see section 1.2.2). Taken in conjunction with the previous experiments, this suggested that the reversed PGK promoter does not play a major role in expression of the *gol* allele. The possibility still exists that expression is at too low a level to allow for detection by the above methods; but it is then questionable whether such a low level of expression would be biologically relevant. This experiment still does not exclude the possibility that the reversed PGK promoter drives expression of an alternative *Brca1* transcript which initiates further downstream than was tested by these 5' RACE reactions; but in that case, the alternative transcript should be at least 1 kb smaller than the wildtype transcript, and thus detectable on a Northern blot.

6.2.6 Analysis of *Brca1* expression : Northern blot and semi-quantitative RT-PCR

Northern blot analysis of *Brca1* expression in ES cells carrying the *gol* allele was performed using two cDNA probes; one containing exons 6-10, and another exons 22-24. This latter, 3' probe was expected to hybridize to both endogenous and alternative transcripts.

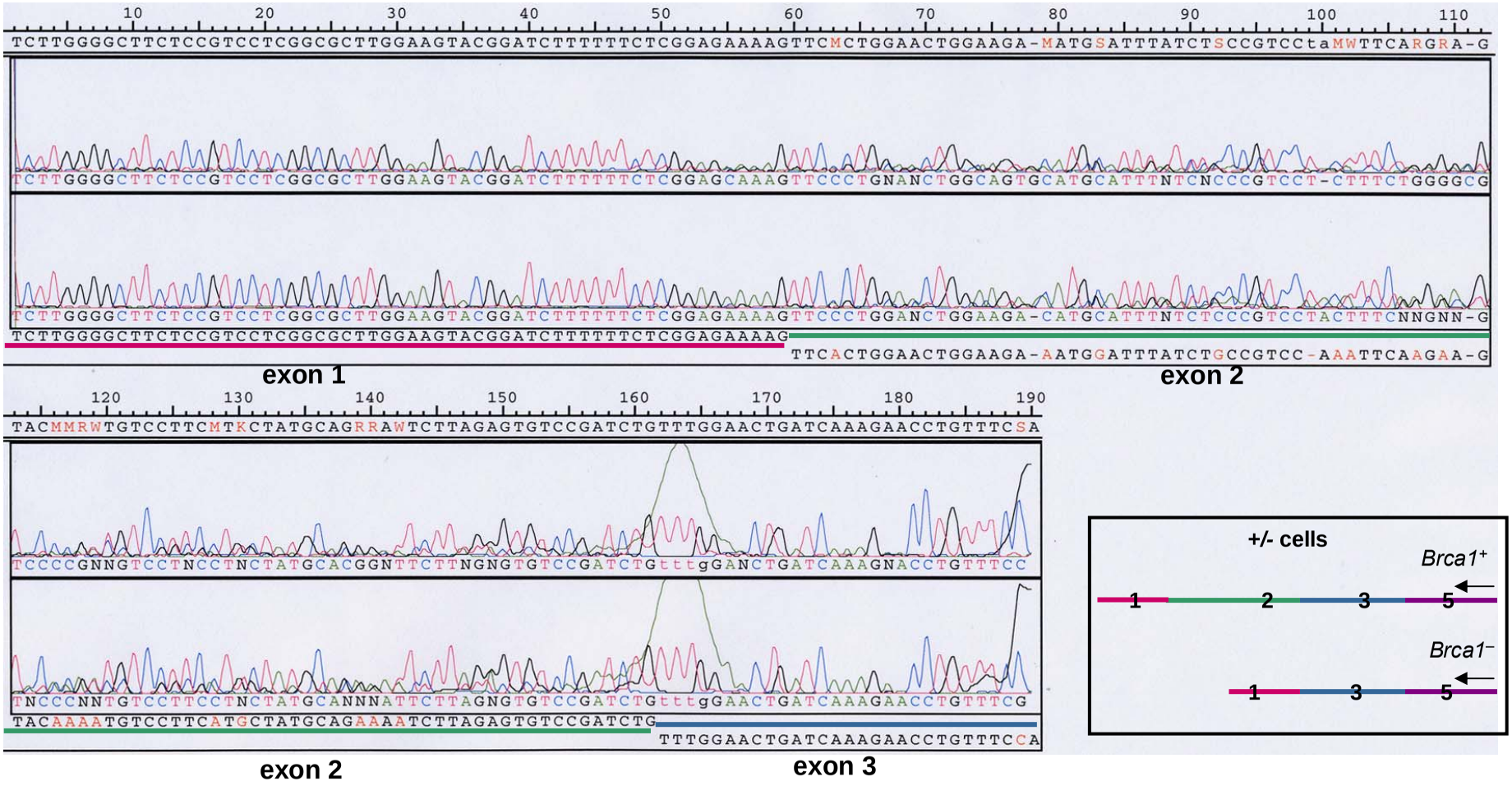


Figure 6.6: Direct sequencing of the +/– 5' RACE product pool. a. Sequence of pooled 5' RACE products from +/– ES cell RNA, showing an "interference pattern" at exon 2 (underlined in green). PCR amplification of 5' RACE pools was performed using the Abridged Anchor Primer (AAP) and *Brca1* exon 10R. Products shown here were sequenced using the *Brca1* exon 5 R primer. **Inset:** wildtype and *Brca1*$^{-}$ alleles. An interference pattern is expected to be evident where the two alleles do not overlap exactly (most of exon 2). *Brca1* does not have an exon 4.

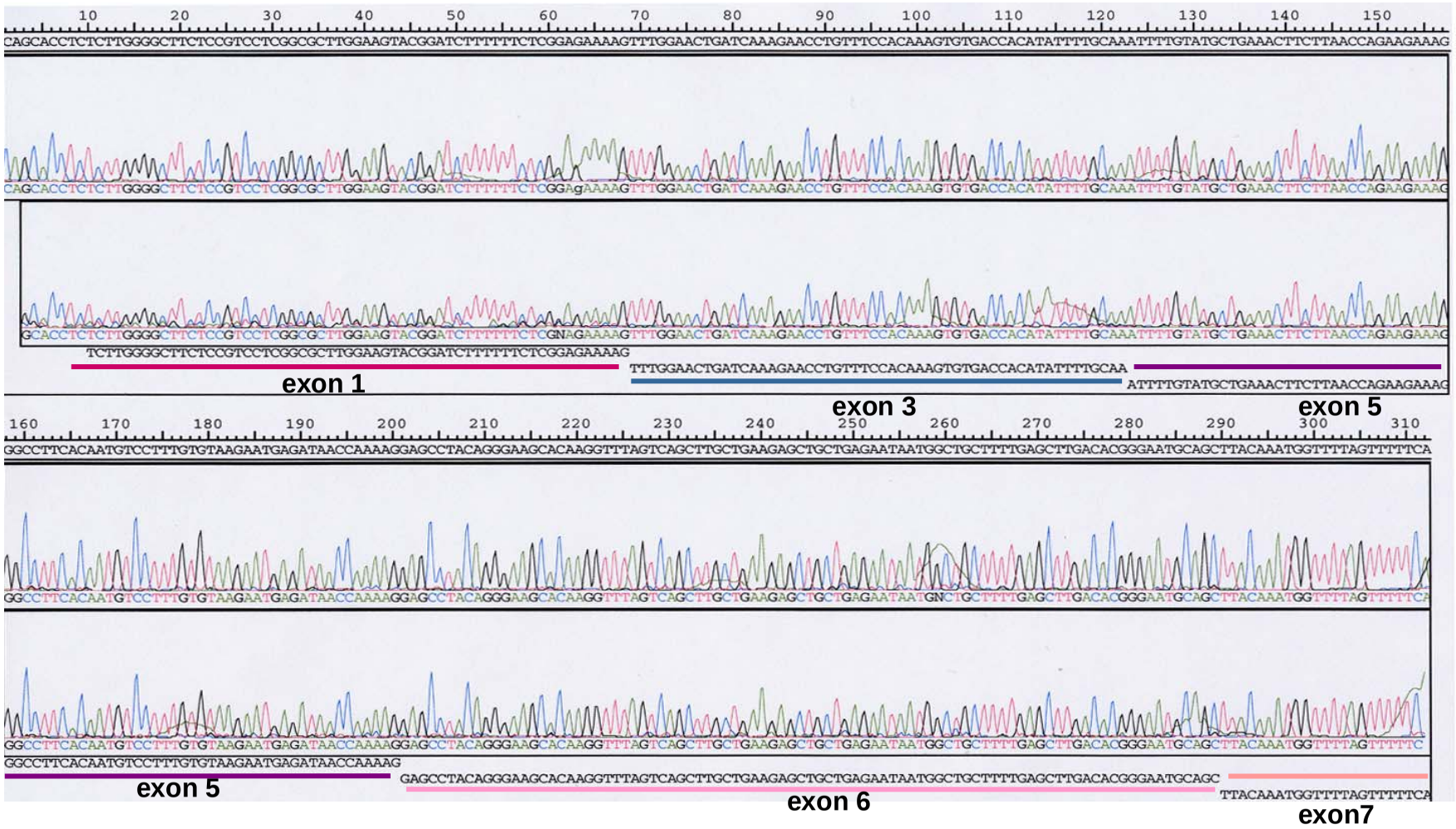
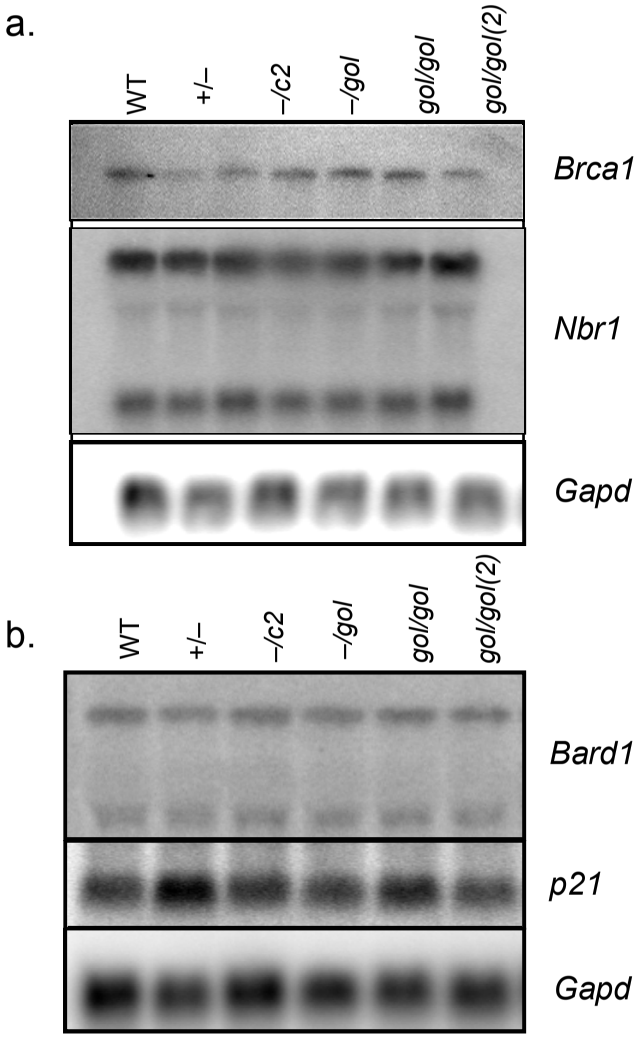


Figure 6.7: Direct sequencing of the *gol/gol* 5' RACE product pool. Sequence of pooled 5' RACE products amplified from *gol/gol* ES cell RNA. No interference pattern is seen at any point up to the exon 6-7 boundary. This sequence shows the exon 1-3 splice pattern of the major transcriptional product of the *gol* allele, *Brca1 ΔX.2*. PCR amplification of 5' RACE pools was performed using the Abridged Anchor Primer (AAP) and *Brca1* exon 10R. Products shown here were sequenced using the *Brca1* exon 7 R primer. *Brca1* does not have an exon 4.

The probe consisting of exons 6-10 appeared to cross-react with the rRNA bands, but only one *Brca1* transcript was detected (data not shown). The 3' probe detected only a ~7.4 kb transcript, corresponding to full-length *Brca1*, in all samples. The level of *Brca1* expression is not appreciably different between wildtype, *+/–,* or *gol/gol* cells after expression is normalized to the level of a *Gapd* loading control (Figure 6.8a and Table 6.2a). The normal *ΔX.11 Brca1* splice isoform was not detected by the 3' probe, although it is detected by RT-PCR (Figure 6.8c). Failure to detect this product by Northern blot may be because the overall level of *Brca1* expression was fairly low.

Semi-quantitative RT-PCR was performed to determine the differences, if any, between the levels of *ΔX.2* and wildtype *Brca1* transcripts in ES cells of various genotypes (Figure 6.9). This assay was performed by generating cDNA from 5 μg of total cellular RNA, making serial dilutions of the cDNA, and performing duplex PCRs using primers in *Brca1* (exons 1 and 6) and *Gapd* (control). Pilot experiments were run in the first instance to determine optimal primer concentrations, annealing temperature, and Mg-ion concentrations for the PCR reactions, then the amount of template and the number of cycles to be used was determined. At the primer concentrations used, neither product reached plateau phase (point at which the amount of PCR product is no longer increase exponentially) before 33 cycles, and reactions were thus run for 31 (at 1:40 and 1:80 dilutions) and 33 (at 1:40, 1:80, and 1:160 dilutions) cycles. Reactions were done in triplicate, and two separate RNA samples of each genotype were subjected to analysis. Figure 6.9a shows a representative gel of PCR products and controls from various genotypes. Figure 6.9b shows a graphical representation of the results from an averaging of all experiments.

Two points are evident: one, the overall amount of *Brca1* mRNA, as normalized to the *Gapd* loading control, is very similar across all genotypes. Second, while the amount of mutant (*ΔX.2*) transcript differs only slightly between the *+/gol* and *+/–* cell lines, the ratio of the mutant product to the wildtype product is significantly different between the two lines. The ratio of



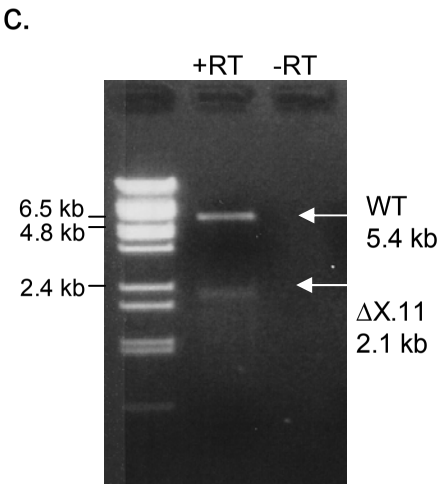


Figure 6.8: Expression levels of *Bard1, Brca1, p21, Nbr1*, and *Gapd* mRNAs. a. Northern blot of *Brca1* (3' cDNA probe comprising exons 22-24) and *Nbr1* expression compared to a *Gapd* loading control. **b.** Northern blot of *Bard1* and *p21* expression levels compared to a *Gapd* loading control. Probes were hybridized serially, after stripping, to the same blot. 20 μg of total ES cell RNA was loaded for each sample. **c.** RT-PCR of wildtype ES cell RNA, using PCR primers in *Brca1* exons 2 and 24. The smaller *ΔX.11 Brca1* isoform and full-length transcript are both detected.
WT=wildtype. See Table 6.2 for transcript quantitation.

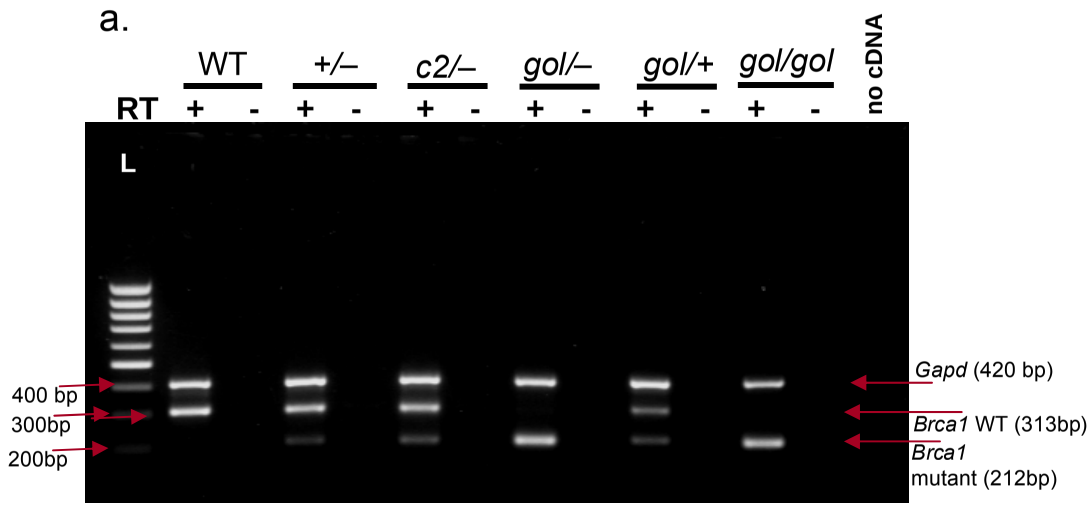
Table 6.2: Northern blot analysis: transcript intensities.
a. Total pixel volume for transcript-related bands from the Northern blot shown in Figure 6.2a. **b.** Total pixel volume for transcript-related bands from the Northern blot shown in Figure 6.2b. Pixel volume was measured in ImageQuant (Molecular Dynamics) with background subtracted. The relative volume of each transcript compared to a *Gapd* loading control is shown in the last two rows of each table. *Nbr1* and *Bard1* both have two transcripts; their values were averaged before being compared to the loading control.

a.

ES cell line:	wildtype	+/–	c2/–	–/gol	gol/gol	gol/gol(2)
Transcript						
Gapd	6.86	5.63	6.52	5.78	5.81	5.90
Nbr1 (average)	64.11	56.65	62.17	48.74	51.46	54.83
Brca1	3.98	2.96	3.37	3.88	4.01	3.93
Nbr1/Gapd	10.04	10.81	10.36	9.60	9.86	10.22
Brca1/Gapd	0.58	0.52	0.52	0.67	0.69	0.67

b.

ES cell line:	wildtype	+/–	c2/–	–/gol	gol/gol	gol/gol(2)
Transcript						
Gapd	7.48	6.47	7.61	6.83	6.71	7.06
Bard1 (average)	2.15	1.76	2.43	2.13	2.18	2.02
p21	13.61	19.90	14.90	12.92	15.64	11.94
Bard1/Gapd	0.29	0.27	0.32	0.31	0.33	0.29
p21/Gapd	1.82	3.08	1.96	1.89	2.33	1.69



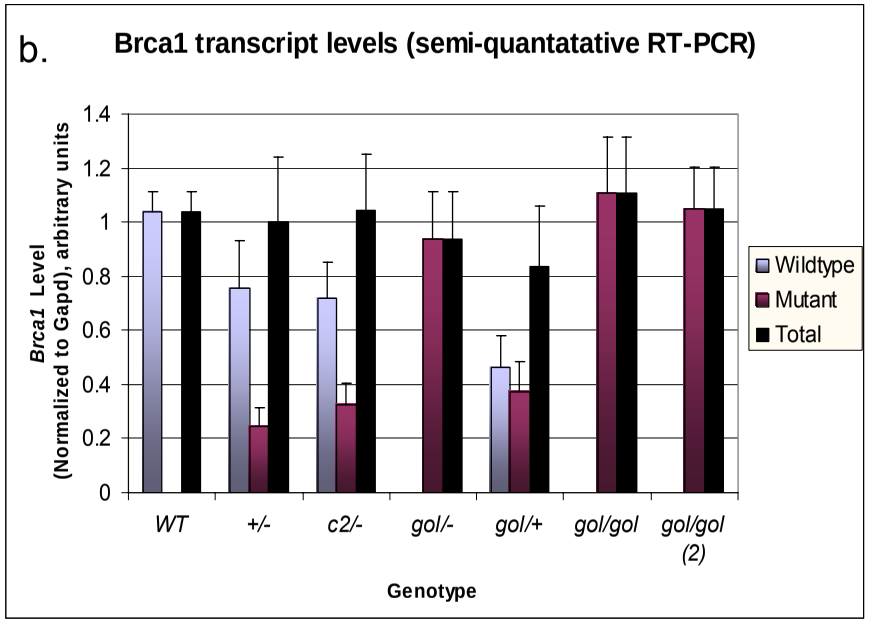


Figure 6.9: Semi-quantatative RT-PCR of *Brca1*.
a. Representative PCR gel of semi-quantataive PCR run on cDNA of the indicated genotypes. - RT indicates samples without reverse transcrptase added. See materials and methods for complete methods; this gel depicts samples from a 33-cycle PCR using PCR primers in *Brca1* exons 1 and 6 (lower two bands) and *Gapd* control primers (top band) using a 1:40 dilution of cDNA.
b. Gradphical representation of wildtype and mutant Brca1 levels (normalized to the Gapd control). The overall level of Brca1 is indicated by the black bar.
Error bars represent one standard deviation of all samples.
L=ladder, RT=reverse transcriptase.

mutant:wildtype product in *+/gol* cells is 1:1.2, while the ratio is 1:3.1 in *+/–* cells. This suggests that while there may be an overall control on the amount of *Brca1* transcript in cells (regardless of genotype), the ratio of the products appears to be the only real difference between the two mutant cell lines. This will be discussed further in light of data presented in the next sections.

6.2.7 Northern blot analysis of other genes

As no change in *Brca1* expression was observed between *gol/gol* and wildtype ES cells, the expression level of three other genes with known links to *Brca1* were assessed in *gol/gol* ES cells (for all three, transcript level was compared to that of a *Gapd* control). *Brca1* shares a bidirectional promoter with the gene *Neighbour of Brca1 1* (*Nbr1*); it was hypothesized that a change in the expression of this gene might contribute to or cause the phenotype of the *gol/gol* cells. However, the expression of this gene is not changed in *gol/gol* ES cells (Figure 6.8a and Table 6.2a).

In *Xenopus* (frog) embryos, following antisense-mediated depletion of *xBRCA1* (the *Xenopus* homologue of *BRCA1*), the level of xBARD1 (the *Xenopus* homologue of BARD1) protein is decreased, and overexpression of either *xBRCA1* or *xBARD1* appears to result in stabilization of the other protein (Joukov, 2001b). McCarthy *et al.* have shown that a similar mutual protein-level control may exist in mice, as *p53*$^{-/-}$, *Bard1*$^{-/-}$ embryos have a decreased amount of Brca1 protein, while *p53*$^{-/-}$, *Brca1*$^{-/-}$ embryos have a decrease in the amount of Bard1 protein (McCarthy, 2003). In the *Xenopus* study, the mRNA levels of the two genes were unaffected, but mRNA levels were not assessed in the mice. To rule out an effect of a change in *Bard1* expression on the phenotype of *gol/gol* cells, *Bard1* expression was assessed by Northern blot, but no change in *Bard1* expression was observed in *gol/gol* ES cells compared to the wildtype control (Figure 6.8b and Table 6.2b).

Overexpression of *p21* has been observed in heterozygous *Brca1* knockout mice, and in mice either homozygous or heterozygous for an exon 11 truncation (Hakem, 1996; Ludwig, 2001). Assessment of *p21* expression

levels in ES cells generated in this study indicated that +/– cells and one of two *gol/gol* cell lines have a slight increase in *p21* expression compared to the wildtype sample (Figure 6.8b and Table 6.2b). Overall, the level of *p21* appears high in these ES cells (irrespective of genotype), countering previous reports of low *p21* expression in ES cells generally (Savatier, 1996). The reason for this is not clear, but could be attributed to a long exposure of the blot.

6.2.8 Brca1 protein is more abundant in cells carrying a *gol* allele

The amount of Brca1 protein present in ES cells of various genotypes was assessed by Western blot analysis. Detection of Brca1 protein was difficult in extracts from wildtype cells, or cells carrying *Brca1⁻* or *c2* alleles, but much easier in those from cells carrying a *gol* allele (Figure 6.10a). A twin Western blot probed with an antibody against α-tubulin demonstrates that roughly equal amounts of protein are loaded for each sample and that degradation of the protein samples does not appear to have occurred (Figure 6.10b). To further provide evidence of roughly even loading, what are presumably non-specific bands at the bottom of the Brca1 blot are also shown (Figure 6.10c). Brca1 protein may be more difficult to detect in wildtype cells due to a combination of low expression and rapid turnover. Blots were detected by ECL.

6.2.9 Alternative reasons for *gol* transcript being more highly expressed and a possible role for suppressors or enhancers of transcription

One striking feature of Figure 6.10 is the apparent difference in protein levels in cells carrying the *gol* allele compared to cells carrying the *Brca1⁻* allele. Expression of the *Brca1⁻* allele does not appear to increase the level of Brca1 protein present in the cell as dramatically as expression of the *gol* allele does. However, this observation re-poses the question of how the *Brca1⁻* and *gol*

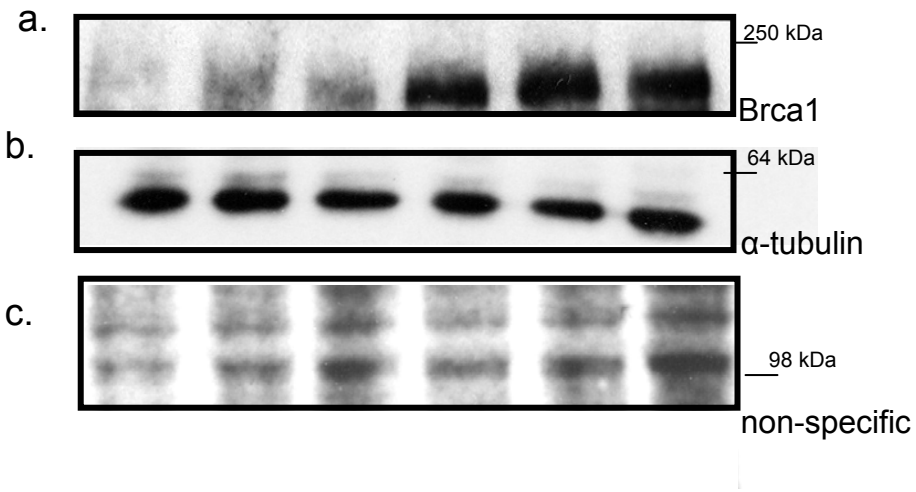


Figure 6.10: An increased amount of Brca1 protein in cells carrying a *gol* allele. a. Western blot of 30 μg total protein isolated from ES cells of the indicated genotypes, detected using the GH118 Brca1 antibody. ECL was used to detect bands. **b.** Duplicate Western blot run with the same samples and at the same time, detected using an α-tubulin antibody. Bands were detected using ECl. c. Secondary bands on from **a,** apparently non-specific, provided as a secondary loading control.
Molecular weights of the SeeBluePlus2 ladder (Invitrogen) are indicated.

alleles differ if the reversed PGK promoter is not involved and the amount of *Brca1* mRNA is similar in *+/–* and *gol/gol* cells.

Mis-expression of *Brca1* in a cell is deleterious; cell cycle arrest and subsequent apoptosis result from overexpression, and loss of Brca1 protein or mRNA expression (as in mouse knockouts or human breast cancers) is also harmful or fatal (Thompson, 1995; Hakem, 1996; Holt, 1996; Liu, 1996; Ludwig, 1997; Sourvinos and Spandidos, 1998). Control of Brca1 at both the protein and mRNA levels is known to occur, allowing differential expression of *Brca1* at different times of development, in response to DNA damage, or at certain stages of the cell cycle (Marquis, 1995; Ruffner and Verma, 1997; Scully, 1997b). As *gol/gol* cells contain a larger amount of Brca1 protein – but not mRNA – than wildtype cells, it seems reasonable to infer that the steady-state level of the different *Brca1* transcripts in ES cells is regulated differently in *+/–* and *+l/gol* cells. Semi-quantitative RT-PCR has shown than the difference in mutant transcript level between *+/–* and *+/gol* cell lines is fairly subtle – the *+/gol* cell line expresses slightly more mutant transcript, and the ratio of wildtype:mutant transcript in this cell line is close to 1:1. However, this assay also suggests that the overall level of *Brca1* is regulated, regardless of genotype, and shows that mutant transcript can be expressed at normal (comparable to wildtype) levels in *gol/gol* or *–/gol* cells. Coupled with the different amount of Brca1 protein detected in *+/–* cells compared to *+/gol* cells, these assays suggest that the proportion of *ΔX.2 Brca1* transcript in relation to the total (or wildtype) amount of *Brca1,* while admittedly subtle, is important. The caveat must be added that a semi-quantitative assay, while more accurate than straight RT-PCR, is not a fully quantitative assay, and could be further supported by one of these assays, such as a TaqMan real-time PCR approach or an RNase protection assay. The advantages to a real-time PCR method include increased accuracy stemming from lack of the need to load samples on gels for analysis (possibly subject to pipetting errors), and the ability to monitor amplification over several cycles, rather than running a set number of cycles before analysis. Further, the mutant and wildtype *Brca1* transcripts could be detected separately by using two assays, which might further increase the accuracy of their quantization. However, semi-

quantitative RT-PCR has been used by a number of labs to determine transcript levels, and when carefully set up and run against TaqMan-type assays, can come very close in accuracy.

What might cause the difference in relative transcriptional amounts? As discussed earlier, the differences between the *Brca1⁻* and *gol* alleles are the selection cassettes (*Hprt* and *Puro*, respectively) and that *gol* lacks ~2 kb more intronic sequence than does *Brca1⁻* (*Brca1⁻* replaces 700 bp of *Brca1* genomic sequence, while *gol* replaces 2.7 kb; Figure 6.1). Therefore, one hypothesis is that either the *Puro* selection cassette in the *gol* allele acts as a transcriptional enhancer, or that an intronic transcriptional repressor normally resides in the intronic region deleted in the *gol* allele. This would help explain why, though the two alleles produce the same transcripts, they are not phenotypically identical. Loss of an intronic suppressor in the *gol* allele is supported by protein data generated from *–/c2* and *–/gol* ES cells. Both the *c2* and *gol* alleles carry the *Puro* cassette, but *c2* has not undergone recombination, so possesses both exon 2 and the surrounding intronic sequence (compare Figure 3.3b and c). Figure 6.10a indicates that *–/c2* cells have protein levels comparable to wildtype cells, while *–/gol* cells have a greater amount of protein than wildtype cells. Further, Figure 6.9 indicates that *–/c2* cells have an amount of wildtype *Brca1* transcript similar to that of *+/–* cells, suggesting that the *c2* allele is still being regulated as a wildtype-like allele. This suggests that deletion of the intronic region may be linked to increased protein levels, although it may also simply reflect the presence of exon 2, and, presumably, normal *Brca1* production. There is the alternative explanation that some type of *trans* effect could be occurring in which the *gol* transcript interferes with the turnover or transcription of the wildtype transcript.

Unfortunately, a reliable prediction program for modifiers of transcription does not yet exist to help confirm this hypothesis. However, intronic modifiers of transcription, both enhancers and repressors, have been widely reported in the literature. For example, Brinster *et al.* generated transgenic mice which expressed the rat growth hormone gene, either with or without introns, under the control of a liver or pancreas-specific promoter. They found that

expression of the transgene was much higher when the introns were present (Brinster, 1988). Additionally, effective expression of both the mouse and human *Hprt* genes depends on the presence of certain introns (Reid, 1990; Magin, 1992).

Regardless of genotype, the level of *Brca1* mRNA is fairly consistent in all cell lines tested in this study. This suggests that the protein translated from the *Brca1 ΔX.2* transcript may be more stable than is wildtype Brca1. This could help explain the larger amount of Brca1 protein detected in cells carrying the *gol* allele compared to the other cell lines (Figure 6.10). Figure 6.11 shows a model which attempts to integrate the data presented in this chapter.

6.2.10 Brca1^{gol} and Bard1 have decreased interaction in a pull-down assay

As described in section 6.2.1, translation of Brca1^{gol} from the *Brca1 ΔX.2* transcript likely initiates at one of the AUG codons in exon 5 or 6. Regardless of which AUG is used, the resulting protein will lack part of the N-terminal RING domain. In previous analyses by other groups, the interaction of BRCA1 and BARD1 was abrogated by certain point mutations in the RING domain of BRCA1; the effect of a larger deletion is likely to be equally detrimental (Wu, 1996; Morris, 2002). To investigate if Bard1 can interact with Brca1^{gol}, glutathione-S-transferase (GST)–Brca1 and myc-Bard1 fusion proteins were generated and co-expressed in mammalian 293T cells. Full-length *Bard1* cDNA was cloned into an N-terminal c-myc–tag vector, while pieces of *Brca1* (from exons 2, 3, 5, or 6 to exon 10) were cloned into an N-terminal GST-fusion vector (Figure 6.12). These four Brca1 fusions mimic, respectively, wildtype Brca1, an exon 2 deletion, Brca1 initiating at the exon 5 AUG, or Brca1 initiating at the first AUG in exon 6 (the latter two begin at the codon following the AUG).

Immunoprecipitation of GST-Brca1 fusions was followed by Western blot analysis using a c-myc antibody to detect the Bard1 fusion, and vice versa. Figure 6.13 demonstrates that, unfortunately, the c-myc antibody cross-reacts

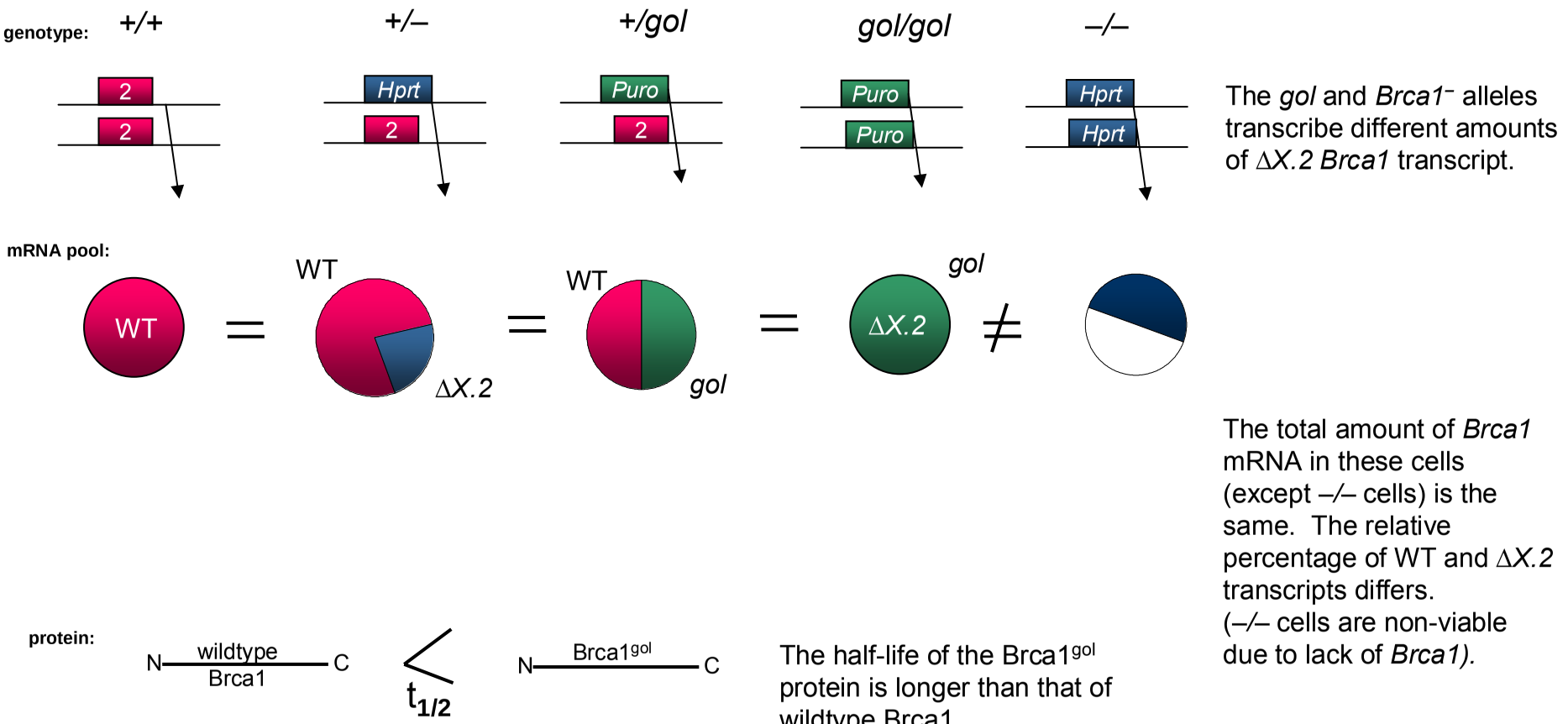


Figure 6.11: Model of differential expression of the *Brca1⁻* and *gol* transcripts. Both the *Brca1⁻* and *gol* alleles produce the *Brca1* Δ*X.2* transcript, but this model suggests that the Δ*X.2 (gol)* product is more abundantly transcribed by the *gol* allele. Although the overall level of *Brca1* mRNA is equivalent in all viable ES cells, a larger proportion of the overall mRNA in *+/gol* cells is comprised of the *Brca1* Δ*X.2* transcript. –/– cells are not viable because they do not produce sufficient *Brca1* transcript. Furthermore, the Brca1 ΔX.2 protein is predicted to be more stable than wildtype Brca1.

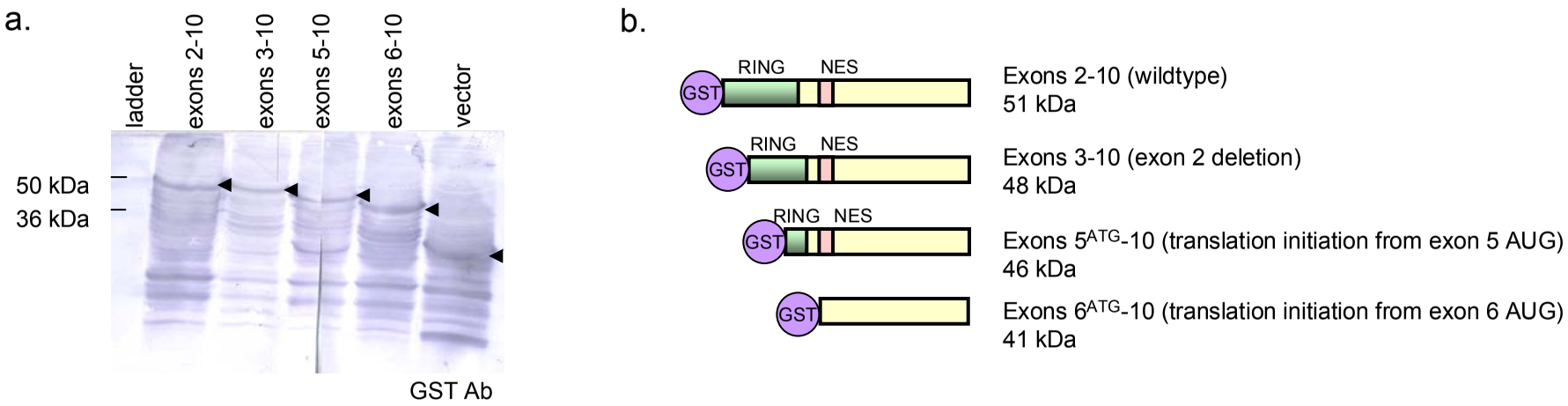


Figure 6.12: GST-Brca1 and myc-Bard1 fusion proteins. a. Western blot analysis of N-terminal GST-Brca1 fragment (exons 2, 3, 5, or 6 to exon 10) fusion proteins; arrows indicate correct products. 30 μg of total protein extract from 293T cells transiently transfected with fusion protein vector or empty vector was loaded per lane. Expected sizes are 51, 48, 46, or 41 kDa, respectively (empty vector, 27 kDa). **b.** Schematic of the four GST-Brca1 fusion proteins and what they mimic. **c.** Western blot analysis of N-terminal myc-tagged, full-length Bard1 (arrow). 30 μg of total protein extract from 293T cells transiently transfected with the myc-Bard1 fusion protein vector was loaded per lane. c-myc antibody was used for detection. Expected size of the fusion protein is ~90 kDa. Approximate molecular weights of the SeeBluePlus2 ladder (Invitrogen) are indicated. Ab=primary antibody used for detection, GST=glutathione S-transferase, NES= nuclear export signal.

with the GST-Brca1 fusions. All four GST-Brca1 fusions are detected by the c-myc antibody following a GST immunoprecipitation (Figure 6.13a, lanes 2-5), and are additionally immunoprecipitated by the c-myc antibody (Figure 6.13b, lanes 3-5). The c-myc antibody does not appear to immunoprecipitate the empty GST fusion vector (Figure 6.13b, lane 2), and the cross-reaction was not abrogated by more stringent washing of the antibody-antigen-bead complex following the immunoprecipitation reaction (done on samples in Figure 6.13b).

Fortunately, myc-Bard1 alone was neither detected nor immunoprecipitated by the GST antibody (Figure 6.13b, lane 6 and Figure 6.13c), so a GST immunoprecipitation could still be used to assess the interaction of myc-Bard1 and GST-Brca1. Figure 6.13a indicates that the GST-Brca1(exons 2-10) fusion protein, which mimics wildtype Brca1, appears to bind strongly to myc-Bard1. A decreased amount of binding is demonstrated by the exons 5-10 (lane 12) construct, and a very faint band can be seen in the exons 6-10 (lane 13) construct. Detection of myc-Bard1 with a c-myc antibody following a c-myc-immunoprecipitation (Figure 6.13a, lanes 6-9) provides a control for the amount of myc-Bard1 which should be detected following immunoprecipitation. This amount is very similar to the amount co-immunoprecipitated with GST-Brca1(exons 2-10) in lane 10. The failure to see any binding in the exons 3-10 lane, which should theoretically resemble the other two mutations, may be because of improper folding of the protein. However, it should be noted that no loading control is used on these blots, leaving interpretation of relative amounts of protein open to some question.

This experiment indicates that Bard1 and Brca1^{gol} are, depending on where the translational start site is, likely to interact at a decreased level. A decrease (vs. abrogation) in binding is somewhat surprising considering the structural data (Figure 6.2) and previous experiments describing the effect of point mutations on the interaction (Wu, 1996; Brzovic, 2001b). However, the reduced amount of binding observed may be due in part to the assay, in which large amounts of protein are transiently expressed in a cell line before the pulldown is performed. It would be worthwhile to attempt to co-

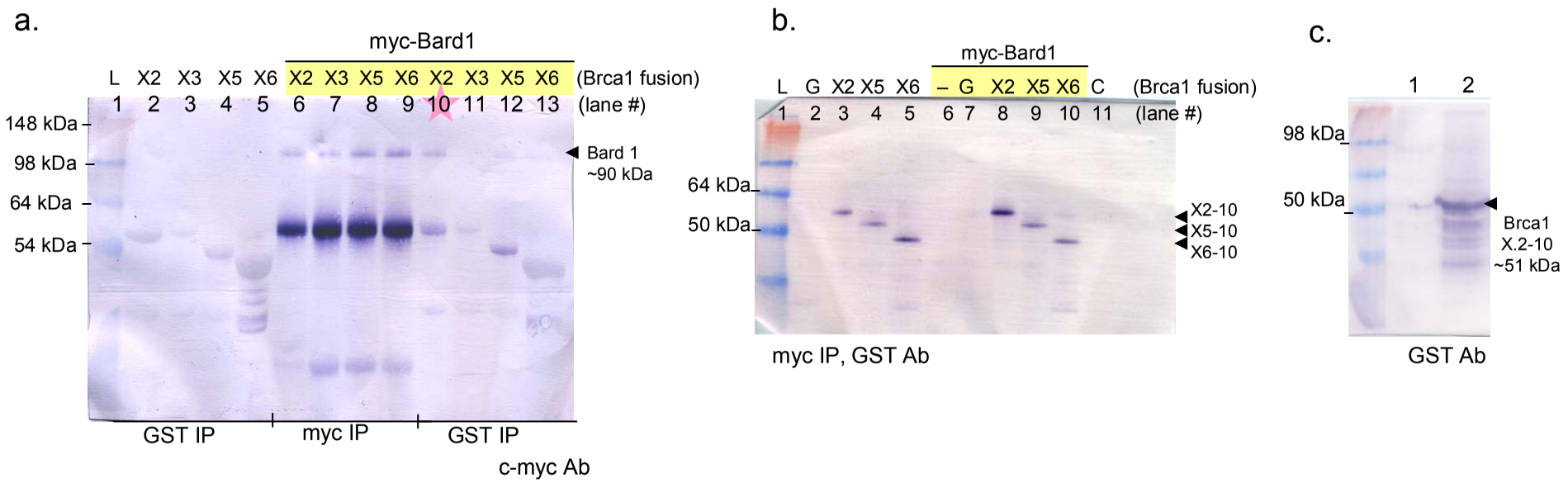


Figure 6.13: Co-immunoprecipitation of GST-Brca1 and myc-Bard1 fusion proteins. a. c-myc-probed Western blot of GST or c-myc immunoprecipitations (IPs) from cells expressing the indicated fusion protein vectors. Lanes 2-5 show that the c-myc antibody (Ab) immunoprecipitates the GST-Brca1 fusion proteins non-specifically. Lanes 6-9 indicate that the myc-Bard1 protein (90 kDa band, arrow) is immunoprecipitated and recognized by the c-myc antibody; the other two bands are the c-myc Ab heavy and light chains, detected by the secondary Ab. Lanes 10-13 indicate that only GST-Brca1(exons 2-10) successfully co-IPs the myc-Bard1 fusion. **b.** GST-probed Western blot of c-myc IPs from cells expressing the indicated fusion proteins. Lanes 3-5 show that the c-myc antibody recognizes the GST-Brca1 fusion proteins (even after more stringent washing), but not the empty GST vector (lane 2). Lane 6 indicates that myc-Bard1 is not immunoprecipitated by the GST antibody. Lane 11 contains extract from non-transfected cells. **c.** GST-probed Western blot. 30 μg of protein from cells expressing only the myc-Bard1 fusion (lane 1) or GST-Brca1(exons 2-10) fusion protein (lane 2, arrow), indicating that myc-Bard1 is not recognized by the GST antibody. Approximate molecular weights of the SeeBluePlus2 ladder (Invitrogen) are indicated. IP=immunoprecipitation, Ab=primary antibody, X=exon, L=ladder, G=GST empty vector.

imunoprecipitate Brca1^{gol} from *gol/gol* ES cells using a Bard1 antibody to confirm that the two can interact *in vivo* when expressed and regulated at normal levels.

Previous *in vitro* studies using transiently expressed BARD1 and BRCA1 have indicated that when the RING domain of BRCA1 is mutated or lost, BRCA1 is found mostly in the cytoplasm, presumably due to the presence of both NLSs and the NES (Fabbro, 2002). The Brca1^{gol} protein localizes to the nucleus and cytoplasm, and its localization is very similar to that of wildtype Brca1. This suggests that, in addition to the loss of the RING domain, the NES may be mutated or lost in Brca1^{gol}, as would be the case if translation initiated from the second downstream AUG. *In vitro* data has indicated that the NLSs coded by exon 11 are sufficient to direct BRCA1 to the nucleus in the absence of the NES (Rodriguez and Henderson, 2000). Although it appears that Bard1 may be able to interact with Brca1^{gol}, it is unknown at present if the lowered amount would be sufficient for all Brca1^{gol} to be properly chaperoned, so the loss of the NES may well be important.

6.3 DISCUSSION

Initially, the structure and viability of the *gol* allele led to speculation that the PGK promoter from the *Puro* selection cassette functioned as a secondary promoter to drive expression of *Brca1* starting from a downstream, in-frame AUG codon. Such downstream AUG codons do exist at the 5' end of Brca1, in exons 5 and 6. However, while the PGK promoter does appear to be able to function bidirectionally in ES cells (Table 6.1), no evidence for its involvement in *Brca1* expression has been observed in the experiments described in this chapter. Reports of aberrant transcripts driven by a reversed PGK promoter are not particularly prevalent in the literature, but in the two cases when the resulting aberrant transcript was sequenced, part of the reversed promoter itself was present in the transcript (Abeliovich, 1992; Scacheri, 2001). No such sequence was observed in transcripts of *gol/gol* ES cells as assessed by Northern blot, RT-PCR, or 5' RACE (Figures 6.5, 6.8, and data not shown).

6.3.1 Transcriptional control of the *gol* and *Brca1*$^{-}$ alleles

While 5' RACE analysis indicated that *gol/gol* cells express both the *Brca1 ΔX.2* isoform, which splices from exon 1 to exon 3 (Figure 3.9) and a smaller novel transcript, the *Brca1 ΔX.2* product is also produced by the *Brca1*$^{-}$ allele, and the smaller transcript was detected in both *+/–* and wildtype samples (Figure 6.5). Northern blot analysis similarly failed to detect a secondary transcript which might have originated outside the region analyzed by 5' RACE (Figure 6.8a). These analyses suggested that the *gol* allele does not produce a unique transcript, at least not at a level detectable by these assays. It is doubtful that Brca1^{gol} is a dominant negative form of Brca1, as *+/gol* and *gol/gol* cells would then be expected to have very similar phenotypes. While these two cell lines both have increased levels of Brca1 protein, *gol/gol* cells are hypersensitive to MMC and γ-irradiation, while *+/gol* cells are not (Figures 6.10, 5.4, and 5.5).

Northern blot analysis and semi-quantitative RT-PCR indicated that *Brca1* mRNA does not appear to be overexpressed from the *gol* allele, as might have been expected (Figure 6.8a, Figure 6.9). In fact, the overall level of *Brca1* mRNA was very similar amongst all the genotypes tested, both by Northern blot, and by semi-quantitative RT-PCR assay (Figure 6.8a and Figure 6.9). Only one group has included *Brca1* expression data when describing their *Brca1* ES cell lines, but in that case, expression of *Brca1* did not appear to differ greatly between wildtype and heterozygous ES cells (Gowen, 1996). This may be a result of regulation of *Brca1* mRNA levels in the cell – although certainly loss of one copy of *Brca1* might lead to loss of up to half the normal gene product. Data from many studies has demonstrated that overexpression or loss of *Brca1* is deleterious (Thompson, 1995; Hakem, 1996; Holt, 1996; Liu, 1996; Ludwig, 1997), and that Brca1 is regulated at both the mRNA and protein level during development, pregnancy, lactation, the cell cycle, and following DNA damage (Marquis, 1995; Ruffner and Verma, 1997; Scully, 1997b). The similarity of *Brca1* expression levels

observed in this study amongst ES cells of different genotypes may be a consequence of such regulation.

The inviability of *–/–* ES cells and mice, but viability of *gol/gol* ES cells suggests that the *Brca1⁻* allele may underexpress Brca1 transcript or protein. In Figure 6.9, it is shown that in *gol/gol* or *gol/–* ES cells, *Brca1* appears to be expressed at levels similar to those detected in wildtype cells. Although the overall *Brca1* mRNA level is also similar in *+/–* and *+/gol* ES cells, the proportion of transcript from the alleles differs (1:3.1 compared to 1:1.2). The absolute amount of mutant product in the two cell lines is only subtly different; the relationship of the wildtype and mutant products is significantly different. This may indicate that some type of *trans* effect regulates the level of wildtype and mutant transcripts in *+/gol* or *+/–* cells (which differs between the *gol* and *Brca1⁻* alleles), or it may reflect a difference in the transcriptional regulation of the *gol* and *Brca1⁻* transcripts. This could result from the differences between the *Brca1⁻* and *gol* alleles at the primary sequence level: the selection cassettes and the amount of intronic region deleted. The *gol* allele may have lost an intronic transcriptional repressor, or the *Puro* selection cassette may act as a transcriptional enhancer. Deletion of a transcriptional repressor seems the more likely explanation, as the *c2* allele also carries the *Puro* selection cassette (but retains the intronic regions) and *–/c2* ES cells, unlike *–/gol* ES cells, do not have more Brca1 protein than wildtype cells (Figure 6.10a), and are not hypersensitive to γ-irradiation (Figure 5.4). A small schematic which sums up this model of relative amounts of transcription from the *Brca1⁻* and *gol* alleles is presented in Figure 6.14.

Many examples of intronic enhancers or suppressors have been reported in the literature (Reid, 1990; Magin, 1992; Ash, 1993; Jonsson, 1994; Oskouian, 1997; Scohy, 2000). Such modifiers have often been identified during studies of promoter regions, and as such are frequently located in the first intron following the translational start site. However, enhancers are found in many locations – in the 5' and 3' UTRs, and within genes; tissue-specific expression of the murine gene *Pax6* in the iris and amacrine cells appears to be regulated by an enhancer in intron 4 (Xu, 1999a). Brinster *et al.* have

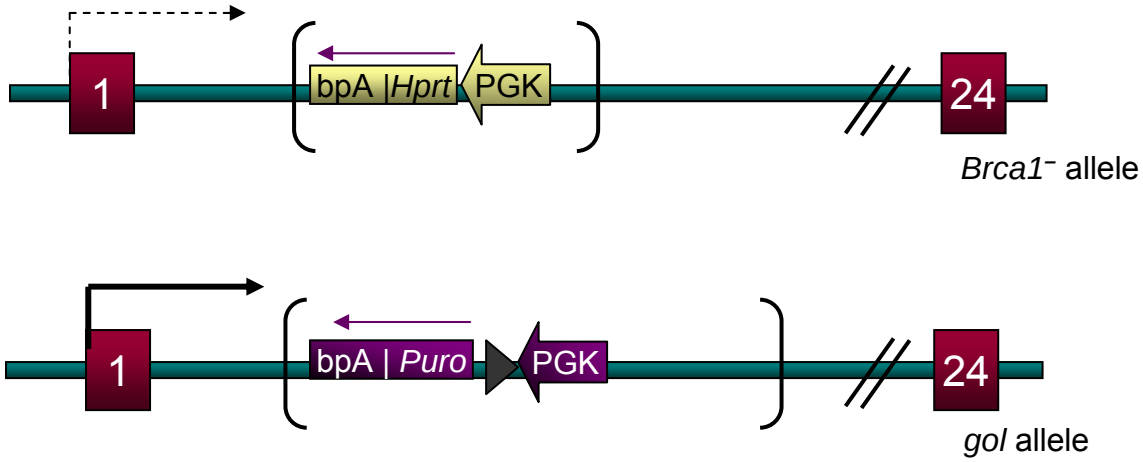


Figure 6.14: The *gol* and *Brca1*⁻ alleles of *Brca1*, model of transcription. Model of transcription of the *gol* and *Brca1*⁻ alleles of *Brca1.* The amount of genomic DNA deleted in the two alleles is indicated by black brackets. Transcription, as indicated by the arrows, is more robust from the *gol* allele than from the *Brca1*⁻ allele. Different amounts of transcription may be due to loss of a transcriptional suppressor in the area deleted in *gol* but not in *Brca1*⁻, or because the *Puro* selection cassette serves as a transcriptional enhancer.

additionally shown that the expression of intron-containing transgenes in mice is higher than expression of the same transgene without the introns (Brinster, 1988). Additionally, effective expression of both the mouse and human *Hprt* genes depends on the presence of certain introns (Reid, 1990; Magin, 1992). A repressor sequence has been identified in intron 1 of human *BRCA1*; this sequence will repress transcription of a reporter gene to which it is linked. However, a similar repressor sequence could not be identified in introns 1 or 2 of murine *Brca1* (Suen and Goss, 2001). A mouse-human intron 2 alignment did identify two regions of homology, but both are located downstream of the *gol* deletion.

Although the difference in mRNA levels in the *+/–* and *+/gol* cell lines is subtle, it does appear to have an effect on the cells, and the amount of protein in the two cell types does appear to differ. Given the small differences seen in the semi-quantitative RT-PCR assay, it is desirable to move to a more quantitative method, such as real-time PCR (as discussed earlier). The semi-quantitative RT-PCR assay relied on running aliquots of reactions on agarose gels followed by analysis of the resulting digital images. Real-time PCR reduces pipetting/analysis errors, and also is able to measure the amount of product per cycle instead of using a "snapshot" of the reaction at one or two points, and should reduce the sample-to-sample variability. Furthermore, a TaqMan assay can be defined that measures the mutant and the wildtype products separately, which may have a bearing on the analysis. Alternatively, an RNase protection assay involving exons 1-3 could be used to assess the relative amounts of transcript produced from the wildtype and mutant alleles in *+/gol* and *+/–* cells. The results of either assay should help generate a more accurate model of transcription of *Brca1* in cells carrying the *gol* allele.

6.3.2 Regulation of the level of Brca1^{gol} protein

One consequence of expression of *Brca1* from the *gol* allele appears to be a greater amount of Brca1 protein. As *gol/gol* cells do not overexpress *Brca1* mRNA, the greater level of protein may indicate that the Brca1^{gol} protein has a longer half life or is translated more efficiently than wildtype Brca1. Increased

stability of Brca1^{gol} was discussed in Chapter 5 as a possible explanation for the presence of aggregates of Brca1 protein in *gol/gol* cells both before and after γ-irradiation (although the presence of aggregates may also be explained by an antibody or cell-based artifact).

While the hypothesis that the region deleted in Brca1^{gol} contains an instability sequence is logical, prediction programs suggest that the putative Brca1^{gol} protein is not more stable than wildtype Brca1, and that the N-terminus of Brca1 contains no PEST domains (protein domains enriched in proline (P), glutamic acid (E), serine (S), and threonine (T), which may serve as proteolytic signals (Rechsteiner and Rogers, 1996; Bioinformatics1, 2003; Rogers, 2003)). However, prediction programs are limited by available information, and the presence of other instability sequences was not investigated.

An alternative explanation for the increased amount of Brca1 protein in *gol/gol* cells may involve the decrease in the Brca1-Bard1 interaction in these cells. In human cells, the BRCA1/BARD1 heterodimer is known to undergo autoubiquitination, a modification postulated to stabilize both components of the heterodimer, suggesting that free BRCA1 or BARD1 is less stable than the heterodimer (Chen, 2002; Mallery, 2002). Additionally, mutual control of Brca1 and Bard1 protein levels has been suggested by experiments involving *Bard1* and *Brca1* knockout mice or *Xenopus* embryos following antisense-mediated depletion of xBRCA1 or xBARD1. These experiments indicated that loss or depletion of one protein results in what appears to be a reciprocal decrease in the amount of the other protein, while overexpression of human or *Xenopus BARD1* or *BRCA1* in cultured human 293T cells results in increased stability of the other protein (Joukov, 2001a; Joukov, 2001b; McCarthy, 2003). This appears to contrast with data from *gol/gol* cells, in which the mutant Brca1^{gol} protein is more abundant or stable than wildtype Brca1 (despite being less prone to heterodimerization with Bard1), but the two observations may be in agreement. Mutual protein-level control may involve the less-stable free form of a more abundant partner stabilizing the second protein, in an attempt to gain a binding partner. Therefore, in the absence of

Brca1, Bard1 would appear to be less abundant than normal, because it is present only in its less-stable free form. In *gol/gol* cells, where the Brca1^{gol} protein is generated but Bard1 interaction is decreased, free Bard1 may stabilize Brca1^{gol} to try and find a binding partner. This could explain the increased amount of Brca1 protein detected in cells carrying the *gol* allele. More experimentation will be required to resolve this issue, and the use of *gol/gol* cells may provide a good tool for further investigation into the control of Brca1 protein levels in the cell.

6.3.3 Bard1 and Brca1

The *gol* allele may prove to be a useful model for studying the Brca1-independent functions of Bard1. Bard1 appears to be necessary for a DNA damage-induced pause in transcriptional processing (Kleiman and Manley, 1999; Kleiman and Manley, 2001), and may itself be a tumour suppressor, though little evidence to support this hypothesis has been reported to date (reviewed in Irminger-Finger and Leung, 2002). Although it has been suggested that not all cellular Bard1 interacts with Brca1 (Chiba and Parvin, 2002), the pivotal role of Bard1 as a Brca1 binding partner and nuclear chaperone (and, now, the role of Brca1 as a Bard1 chaperone (Rodriguez, 2004)) has so far precluded study of its other roles. For instance, the *Bard1* knockout mouse is phenotypically identical both to a *Brca1* knockout mouse and to *Brca1/Bard1* double knockout mice (McCarthy, 2003). The availability of a form of Brca1 which does not interact at normal levels with Bard1 but does localize to the nucleus may help to uncover the other functions of Bard1, as well as to reveal the importance of other roles of the Brca1/Bard1 heterodimer, such as its E3 ubiquitin ligase activity. To the knowledge of this author, the *gol* allele is the only allele of *Brca1* which would allow this topic to be investigated without introducing protein into the cell by artificial expression. Additionally, it would be interesting to pair the *gol* allele with the published *Bard1* knockout allele.

In summary, the *Brca1*$^{-}$ and *gol* alleles, though similar, have very different effects on the cells which carry them. The *gol* allele may serve as a very

useful model for uncovering both the Brca1–independent functions of Bard1 and for investigation of the roles of the Brca1/Bard1 heterodimer.