Results

Chapter 1

Effect of H19-ICR insertion on transcription at the TCRβ locus
Introduction

Transcriptional insulators prevent enhancer-promoter interaction and hence block enhancer-mediated transcriptional activation of the promoter. *H19-ICR*, the imprint control region at the *H19-Igf2* locus, acts as an enhancer blocking insulator which prevents communication between endodermal enhancers and Igf2 gene promoters. While enhancer blocking activity of the *H19-ICR* has been shown at the endogenous position, its ability to act as an enhancer blocking insulator at a heterologous position remains to be tested in an unambiguous manner. For this purpose, using a locus with the organization of transcription units similar to that of the *H19-Igf2* locus was considered to be a useful approach. Keeping these considerations in view, murine TCRβ locus was chosen as the heterologous position in the present study where the enhancer blocking activity of the inserted *H19-ICR* was tested. Like the *H19-Igf2* locus, the 3’ end of the TCRβ locus has two transcription units TRB-DJC1 and TRB-DJC2 (with a shared downstream enhancer, Eβ), which generate germline transcripts in a developmentally regulated manner. Previous work in our lab had led to the generation of genetically manipulated mice in which the *H19-ICR* was targeted at the TCRβ locus between the TRB-DJC1 and TRB-DJC2 clusters. The mutation thus generated, called TCRins, was expected to be useful for testing the enhancer blocking activity in the heterologous context of TCRβ locus.

Strategy for analysis

The TCRβ locus spans approximately 650 kb and comprises of 35 TRB-V gene segments, two TRB-DJC clusters [driven by promoters, PDβ1 and PDβ2] and an enhancer, Eβ (Fig. 6a). In the TCRins allele (Fig. 6b), the *H19-ICR* is positioned
Fig. 6. Schematic diagram of the endogenous and genetically manipulated TCRβ alleles. The variable, TRB-V (blue bars), diversity, TRB-D (orange bars), joining, TRB-J (purple bars) and constant, TRB-C (grey boxes) gene segments are shown in each case. The TRBV1 gene segment is located 150 kb upstream of the TRB-V2 to TRB-V30 gene segments. A 150 kb stretch of inactive trypsinogen genes is found between TRB-V1 and TRB-V2 gene segments. The TRB-V2 and TRB-V30 gene segments are spread in a 250 kb region. Another 250 kb stretch of inactive trypsinogen genes is located between TRB-V2 to TRBV30 and TRB-DJ1 cluster. The trypsinogen genes are not shown. The two DJC clusters represent two transcription units which are regulated by separate promoters, PDβ1 and PDβ2, respectively. The enhancer Eβ controls the 21 kb region (drawn to scale) encompassing the DJC1 and DJC2 clusters. (a) Wild type [+] allele, (b) TCRins mutant allele showing the inserted 2.2 kb H19-ICR region. The four pink bars within the H19-ICR denote the four CTCF-binding sites and (c) TCRβdel allele showing a 15 kb deletion such that regions from TRB-J1.3 to TRB-C2 are replaced by a neomycin resistance gene (neo') shown as white box.
such that the TRB-V1 to TRB-V30 gene segments and TRB-DJC1 cluster are located upstream to the insulator insertion, while TRB-DJC2 cluster, enhancer E\(\beta\) and TRB-V31 gene segment are downstream to the insertion. The germline transcription and D-to-J recombination at the TRB-DJC1 cluster is driven by an interaction between its promoter, PD\(\beta\)1 and the downstream enhancer, E\(\beta\). Similarly, an interaction between PD\(\beta\)2 and E\(\beta\) is responsible for the germline transcription and D-to-J recombination at the TRB-DJC2 cluster. Like all enhancer blocking insulators, the \(H19-ICR\) acts in a position-dependent manner at the endogenous locus such that it is only functional as an insulator when present between the enhancer and promoter. Hence, in case of the TCRins mutants, the inserted \(H19-ICR\) could potentially interfere with the interactions between the promoter PD\(\beta\)1 of the DJC1 cluster and E\(\beta\) as it has been placed between the two regulatory elements. On the other hand, the inserted \(H19-ICR\) is not present between the promoter of the second cluster, i.e. PD\(\beta\)2 and E\(\beta\) and hence was not expected to affect their interaction.

All the mice samples used in the present study had TCR\(\beta\)del (Fig. 6c) as the background allele (Mombaerts \textit{et al.}, 1991). The TCR\(\beta\)del allele has a 15kb deletion such that it lacks major part of the \(TCR\beta\) locus after the TRB-J1.2 gene segment and does not exhibit recombination (Mombaerts \textit{et al.}, 1992). Usage of TCR\(\beta\)del allele as the background allele simplified the analysis since the results obtained for mice with either the TCRins mutant allele or the wild type allele could be directly compared without the interference of the second allele.
In the endogenous context, the *H19-ICR* is differentially methylated and is functional as an insulator only in the maternal allele. Therefore, parent of origin specific matings were set up between TCRins/+ and TCRβdel/TCRβdel mice. Thus, the TCRins allele was inherited in the progeny either maternally [TCRins/TCRβdel, referred to as the maternal TCRins mutants or $M^m$ in the present study] or paternally [TCRβdel/TCRins, referred to as the paternal TCRins mutants or $M^p$ in the present study] with +/TCRβdel \{C$^m$\} and TCRβdel/+ \{C$^p$\} as their respective littermate controls. The mice bearing these genotypes were used for analysis. It is noteworthy here that all of these TCRins mice were found to be healthy and did not possess any phenotypic abnormalities indicating that the insertion of *H19-ICR* insulator did not lead to any growth defects.

**Effect of *H19-ICR* insertion on overall T cell development**

Eβ-dependent germline transcription and recombination is essential for development of T cells (Bouvier et al., 1996; Bories et al., 1996). Due to the insertion of *H19-ICR* insulator in the TCRins mice, the Eβ-mediated regulation of the *TCRβ* locus was likely to get affected. Therefore, it was important for the present study to first determine whether the introduction of the *H19-ICR* at the *TCRβ* locus interferes with the overall T-cell development. The total number of thymocytes in TCRins mutants was found to be comparable to that of their littermate control mice. Under the normal conditions in which the mice were bred, the mutant mice were not found to be more prone to infections compared to their littermate controls.
The early stages of T cell development involve the Double Negative (DN) stage where both CD4 and CD8 cell surface co-receptors are not present (Godfrey et al., 1993). This stage is then followed by the Double Positive (DP) stage, characterized by the presence of both CD4 and CD8 co-receptors (CD4⁺CD8⁺). Subsequently, the thymocytes progress to the Single Positive stage in which either CD4 or CD8 co-receptor is expressed (called CD4-SP or CD8-SP respectively). To determine whether the insertion of H19-ICR was interfering with the normal thymocyte development, developmental profiles of thymocytes were analysed using CD4 and CD8 surface expression. Flow cytometric profiles for CD4 versus CD8 surface antigens of the total thymocytes (Fig. 7a, left panel) from wild type [+/+] mouse showed a normal developmental profile with DN (CD4⁻CD8⁻), DP (CD4⁺CD8⁺), CD4-SP (CD4⁺CD8⁻) and CD8-SP (CD4⁻CD8⁺) thymocytes, where the majority (~80%) of thymocytes were in the DP stage.

According to earlier reports, the thymocyte development profile of total thymocytes from TCRβdel/TCRβdel mice showed decreased percentage of DP thymocytes and consequently the percentage of DN thymocytes was found to be increased. Further, a few CD4-SP and CD8-SP thymocytes were observed, most of which were γδ T cells, while the rest were suspected to be intermediate cells on the way to become DP cells (Mombaerts et al., 1991; Mombaerts et al., 1992). Analysis of the thymocytes from the TCRβdel homozygous mutant mice [TCRβdel/TCRβdel] in the present study showed an altered thymocyte development profile (Fig. 7a, right panel) compared to the wild type [+/+] mouse (Fig. 7a, left panel) that was entirely consistent with the
Fig. 7. Thymocyte development profiles of mice. Flow cytometric analysis was performed after staining the thymocytes with antibodies against CD4 and CD8 surface co-receptors. (a) Left panel shows the development profile for wild type [+/+] while the right panel shows the profile for TCRβdel homozygous mice [TCRβdel/TCRβdel] (b) Upper left panel shows the development profile for maternal TCRins mutant, M^m while the upper right panel shows the profile for maternal control, C^m mice. The lower left panel shows the development profile for paternal TCRins mutant, M^p mice while the lower right panel shows the development profile for paternal littermate control, C^p mice. (a) and (b) represent two independent experiments.
previous findings. Most of the thymocytes from these mice were arrested at the DN stage. Only about 15% of the total thymocytes were observed in the DP stage.

The CD4 versus CD8 developmental profiles of the thymocytes from maternal TCRins mutant mice, $M^m$ [TCRins/TCRβdel] were normal and were comparable to those from their control littermates, $C^m$ [+/TCRβdel] (Fig. 7b). The thymocytes from the paternal TCRins mutant mice $M^p$ and their littermate control mice $C^p$ also showed comparable CD4 versus CD8 developmental profiles.

The development of thymocytes in the TCRins mutant mice beyond DN stage to generate DP and eventually SP thymocytes demonstrated the ability of the TCRins mutant allele to undergo somatic recombination and generate a functional TCRβ chain essential for the appropriate thymocyte developmental progression. While the inserted $H19-ICR$ did not completely abolish V-to-DJ recombination or arrest thymocyte development, the interference of $H19-ICR$ with specific aspects of TCRβ regulation i.e., transcription, D-to-J and V-to-DJ recombination could not be ruled out and was investigated subsequently.

**Analysis of enhancer blocking activity of $H19-ICR$ at the heterologous position**

At the endogenous locus, the $H19-ICR$ abrogates enhancer-promoter interaction and reduces transcript levels of the $Igf2$ gene. Considering that the inserted $H19-ICR$ is placed between the promoter PDβ1 and enhancer Eβ at the TCRβ locus, the $H19-ICR$ could be expected to bring about a reduction in the germline transcript levels from the TRB-DJCl cluster. To this effect, the levels of TRB-DJ1 germline
transcripts were assessed by reverse transcriptase PCR (RT-PCR). Primers were
designed (See Appendix for primer sequences) such that the forward primer [M387]
was positioned slightly upstream to TRB-J1.1 gene segment while the reverse
primer [M386r] was designed downstream to TRB-J1.2 gene segment (Fig. 8a). This
design ensured that the TCRβdel allele did not give any readout for the TRB-DJ1
germline transcription analysis owing to the absence of the region corresponding to
the reverse primer in TCRβdel allele (Fig. 8a). A 422 bp fragment was expected to
be amplified in this RT-PCR reaction. The absence of TRB-DJ1 germline
transcription from the TCRβdel allele was confirmed by conventional RT-PCR on
cDNA derived from total thymocytes of samples with different genotypes (Fig. 8b,
upper panel). The TCRβdel homozygous mutant mice [TCRβdel/TCRβdel] did not
show any amplification for TRB-DJ1 transcript. On the other hand, the wild type
[+/+], maternal TCRins mutant [TCRins/TCRβdel], maternal control [+/TCRβdel],
paternal TCRins mutant [TCRβdel/TCRins] and paternal control [TCRβdel/+] all
showed an amplification for TRB-DJ1 transcript. However, it was noteworthy that
although the maternal TCRins mutants or Mm [TCRins/TCRβdel] showed the PCR
amplification, the levels of the amplification observed were less than those for their
respective littermate controls or Cm. The Thy1.2 gene was used as an internal
reference gene in the analysis (Fig. 8b, lower panel).

The observed reduction in conventional RT-PCRs for the TRB-DJ1 transcript levels
in the maternal TCRins mutants was quantitated using SYBR-Green chemistry
based quantitative Real Time RT-PCR. The same primer pair was used [M387, 386r]
for quantitating the TRB-DJ1 transcripts (Fig. 9a). In cDNA derived from total
Fig. 8. Analysis of TRB-DJ1 germline transcription in TCRins and TCRβdel mutant mice. (a) Upper schematic diagram represents TCRins allele with black arrows showing positions of primers, M387 and M386r for detection of TRB-DJ1 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRins allele. Lower schematic diagram represents TCRβdel allele showing only forward primer's (M387) position. The region corresponding to the reverse primer M386r is absent in TCRβdel allele. (b) Gel pictures of RT-PCR analysis for TRB-DJ1 germline transcription (upper panel) and Thy1.2 transcription (lower panel) on the cDNA prepared from total thymocytes of mice bearing different genotypes. NTC stands for No Template Control.
Fig. 9. TRB-DJ1 germline transcription analysis for total thymocytes of maternal TCRins inheritance samples. Quantitative RT-PCR analysis was performed for maternal TCRins mutants, TCRins/TCRβdel \{Mm, pink bars\} and their littermate control mice, +/TCRβdel \{Cm, grey bars\}. (a) Upper schematic diagram represents TCRins allele with black arrows showing positions of primers, M387 and M386r for detection of TRB-DJ1 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRins allele. Lower schematic diagram represents TCRβdel allele showing only forward primer’s (M387) position. The region corresponding to the reverse primer M386r is absent in TCRβdel allele. (b) Relative transcript levels of TRB-DJ1 normalized to Thy1.2 transcription. A total of five samples were analyzed for maternal TCRins mutant \{Mm1 to Mm5\} and maternal control \{Cm1 to Cm5\} mice. Each bar represents mean with ± SEM of measurements made in triplicates.
thymocytes, the TRB-DJ1 germline transcript levels relative to Thy1.2 transcript levels of maternal TCRins mutant mice, $M^m$ [TCRins/TCRβdel] was reduced to an average of about 14% compared to their littermate controls, $C^m$ [+/TCRβdel] (Fig. 9b).

Eβ regulated germline transcription occurs predominantly in the DN2/DN3 stages of thymocyte development. Subsequently, D-to-J recombination eliminates the D-to-J intervening region. An additional loss of D-to-J region occurs due to V-to-DJ recombination. Together the recombination events, owing to their combinatorial diversity, lead to a significant heterogeneity in the gDNA template that may interfere with the transcriptional analysis. Hence, it was considered prudent to extend the analysis on mRNA derived from sorted DN2/DN3 cells. Elimination of DP and SP cells by sorting was expected to minimize the influence of template heterogeneity on TRB-DJ1 germline transcription. The DN2/DN3 thymocytes were sorted in two batches each containing two maternal TCRins mutant samples or $M^m$ [TCRins/TCRβdel] and a single littermate control or $C^m$ [+/TCRβdel] {mutants $M^{m6}$, $M^{m7}$ with control $C^{m6-7}$ and mutants $M^{m8}$, $M^{m9}$ with control $C^{m8-9}$}. The cDNA derived from these sorted DN2/DN3 cells were subjected to the same SYBR-Green chemistry based quantitation using primers M387-386r, as in case of total thymocytes (Fig. 10a). In this case also, the maternal mutants from the sorted DN2/DN3 thymocytes showed a reduction in TRB-DJ1 transcription compared to their littermate controls (Fig.10b).
Fig. 10. TRB-DJ1 germline transcription analysis for sorted DN2/DN3 thymocytes of maternal TCRβs inheritance samples. Quantitative RT-PCR analysis was performed for maternal TCRβs mutants, TCRβs/TCRβdel (M^m, light pink bars) and their littermate control mice, +/TCRβdel (C^m, grey bars). (a) Upper schematic diagram represents TCRβs allele with black arrows showing positions of primers, M387 and M386r for detection of TRB-DJ1 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRβs allele. Lower schematic diagram represents TCRβdel allele showing only forward primer's (M387) position. The region corresponding to the reverse primer M386r is absent in TCRβdel allele. (b) Relative transcript levels of TRB-DJ1 normalized to Thy1.2 transcription. Two sets of maternal mutants {M^m6, M^m7 and M^m8, M^m9} and their respective littermate controls {C^m6-7 and C^m8-9} are shown. Each bar represents mean with ± SEM of measurements made in triplicates.
Together, the TRB-DJ1 germline transcription analysis results from total thymocytes and sorted thymocytes, suggested that, as in the case of the endogenous locus, the inserted \textit{H19-ICR} drastically reduces the level of germline transcription of the TRB-DJ1 in maternal TCRins mutant, M\textsuperscript{m} mice [TCRins/TCRβdel]. To confirm that the reduction in TRB-DJ1 germline transcription is due to enhancer blocking activity of the \textit{H19-ICR}, the position-dependence of the inserted \textit{H19-ICR} was subsequently tested.

\textbf{Position dependence of the enhancer blocking activity of the inserted \textit{H19-ICR}}

Enhancer blocking insulators act in a position-dependent manner implying that the insulator is only functional when placed between the enhancer and promoter. To test the position-dependence of the insulator activity of \textit{H19-ICR} at TCRβ locus, TRB-DJ2 transcript levels were quantified. The TRB-DJ2 transcripts are initiated by PDβ2 which is not separated from Eβ by the inserted \textit{H19-ICR} and hence could be expected to remain unaffected by the \textit{H19-ICR} insertion. Both the forward [M332] and reverse primers [M333r] (See Appendix for primer sequences) used for RT-PCR assay were situated between the TRB-D2 and TRB-J2.1 gene segments (Fig. 11a). The region corresponding to these primers was absent in the TCRβdel allele. Again, the absence of TRB-DJ2 transcription from the TCRβdel allele was confirmed by conventional RT-PCR on cDNA derived from total thymocytes of samples with different genotypes (Fig. 11b, upper panel). As expected, the TCRβdel homozygous mutant mice [TCRβdel/TCRβdel] did not show any amplification for TRB-DJ2 transcript. On the other hand, the wild type [+/+], maternal TCRins mutant or M\textsuperscript{m} [TCRins/TCRβdel], maternal control or C\textsuperscript{m} [+/TCRβdel], paternal TCRins mutant
Fig. 11. Analysis of TRB-DJ2 germline transcription in TCRins and TCRβdel mutant mice. (a) Upper schematic diagram represents TCRins allele with black arrows showing positions of primers, M332 and M333r for detection of TRB-DJ2 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRins allele. Lower schematic diagram represents TCRβdel allele. The TCRβdel allele does not possess region corresponding to both the primers. (b) Gel pictures of RT-PCR analysis for TRB-DJ2 germ-line transcription (upper panel) and Thy1.2 transcription (lower panel) respectively on the cDNA prepared from total thymocytes of mice bearing different genotypes. NTC stands for No Template Control.
or M^P [TCRβdel/TCRins] and paternal control or C^P [TCRβdel/+], all showed an amplification for TRB-DJ2 transcript. The Thy1.2 gene was used as an internal reference gene in the analysis (Fig. 11b, lower panel).

For quantitative RT-PCR, the same set of primers, M332-M333r was used (Fig. 12a). A total of five sets of maternal TCRins mutants \{M^{m1} to M^{m5}\} and their respective lintermate controls \{C^{m1} to C^{m5}\} were analyzed. The levels of TRB-DJ2 transcripts relative to Thy1.2 transcripts in total thymocytes were reduced to an average of about 50% in maternal TCRins mutants \{M^{m}\} compared to their lintermate controls (Fig. 12b). Such a reduction in the TRB-DJ2 transcript levels might be attributed to the silencing activity of the inserted H19-ICR on the downstream PDβ2 promoter. Alternatively, it could be a manifestation of the heterogeneity in the template DNA that arises due to recombination events as described earlier for TRB-DJ1 transcripts.

To distinguish between these two possibilities and understand the reason behind the observed reduction in TRB-DJ2 transcript levels in total thymocytes from maternal mutants, the TRB-DJ2 transcript analysis was performed on sorted DN2/DN3 thymocytes. In the sorted DN2/DN3 thymocytes, a large population of alleles are in the non-recombined state and thus exhibit germline transcription. Similar SYBR Green based chemistry was used for the quantitation of TRB-DJ2 transcripts along with the same primers (Fig. 13a) as those used for cDNA derived from total thymocytes. The DN2/DN3 thymocytes were sorted in two batches each containing two maternal TCRins mutant samples or M^{m} [TCRins/TCRβdel] and a single
Fig. 12. TRB-DJ2 germline transcription analysis for total thymocytes of maternal TCRins inheritance samples. Quantitative RT-PCR was performed for maternal TCRins mutants, TCRins/TCRβdel {Mⁿ, pink bars} and their littermate control mice, +/TCRβdel {Cᵐ, grey bars}. (a) Upper schematic diagram represents TCRins allele with black arrows showing positions of primers, M332 and M333r for detection of TRB-DJ2 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRins allele. Lower schematic diagram represents TCRβdel allele. The TCRβdel allele does not possess region corresponding to both the primers. (b) Relative transcript levels of TRB-DJ2 normalized to Thy1.2 transcription. A total of five samples were analyzed for maternal TCRins mutants {Mᵐ¹ to Mᵐ⁵} and maternal controls {Cᵐ¹ to Cᵐ⁵} mice. Each bar represents mean with ± SEM of measurements made in triplicates.
Fig. 13. TRB-DJ2 germline transcription analysis for sorted DN2/DN3 thymocytes of maternal TCRins inheritance samples. Quantitative RT-PCR was performed for maternal TCRins mutants, TCRins/TCRβdel \{M^{m}, pink bars\} and their littermate control mice, +/TCRβdel \{C^{m}, grey bars\}. (a) Upper schematic diagram represents TCRins allele with black arrows showing positions of primers, M332 and M333r for detection of TRB-DJ2 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRins allele. Lower schematic diagram represents TCRβdel allele. The TCRβdel allele does not possess region corresponding to both the primers. (b) Relative transcript levels of TRB-DJ2 normalized to Thy1.2 transcription. Two sets of maternal mutants \{M^{m6}, M^{m7}, M^{m8}, M^{m9}\} and their respective littermate controls \{C^{m6-7} and C^{m8-9}\} are shown. Each bar represents mean with ± SEM of measurements made in triplicates.
littermate control or C\textsuperscript{m} [+/TCR\textbeta\text{del}] \{mutants M\textsuperscript{m6}, M\textsuperscript{m7} with control C\textsuperscript{m6-7} and mutants M\textsuperscript{m8}, M\textsuperscript{m9} with control C\textsuperscript{m8-9}\}.

The sorted DN2/DN3 thymocytes showed no significant reduction in the TRB-DJ2 transcript levels relative to the Thy1.2 transcript levels in maternal mutants \{M\textsuperscript{m}\} compared to the littermate controls \{C\textsuperscript{m}\}(Fig. 13b). These results indicated that the reduction observed for TRB-DJ2 transcript levels in total thymocytes from maternal TCR\textalpha\textins mutants was due to heterogeneity in the template DNA rather than the possible silencing activity of the inserted \textit{H19-ICR}. Since the TRB-DJ2 transcripts were not reduced in maternal mutants in the sorted DN2/DN3 cells, it is clear that the \textit{H19-ICR} acts as a position-dependent insulator, rather than a silencer, even in the heterologous context of \textit{TCR\textbeta} locus.

To sum up, the inserted \textit{H19-ICR} lies between the promoter PD\beta\text{1} and E\beta and consequently, a reduction was observed in the levels of TRB-DJ1 transcript levels in thymocytes. On the other hand, the inserted \textit{H19-ICR} does not lie between the PD\beta\text{2} and E\beta. Accordingly, TRB-DJ2 transcript levels were not reduced in thymocytes from maternal TCR\textalpha\textins mutants. These results indicate that, like at the endogenous locus, the inserted \textit{H19-ICR} acts as a position-dependent enhancer blocking insulator at the heterologous context of \textit{TCR\textbeta} locus upon maternal inheritance of the TCR\textalpha\textins mutation.
Effect of *H19-ICR* insertion on transcription at the *TCRB* locus in paternal mutants

At the endogenous locus, the paternal *H19-ICR* does not act as a functional insulator and the *Igf2* gene expression occurs from the paternal allele. Extrapolating from the endogenous locus, the inserted *H19-ICR* in the paternal TCRins mutants, M^P [TCRβdel/TCRins] in the present study could be expected to be non-functional as an insulator. Consequently, the TRB-DJ1 transcription would be expected to be unaffected due to the insertion of *H19-ICR*. To analyze this, quantitative Real Time RT-PCR was performed for TRB-DJ1 transcripts using the same primers, M387-M386r (Fig. 14a), used for maternal mutants.

A variation in TRB-DJ1 transcript levels was observed (Fig. 14b) with some paternal TCRins mutants, M^P [TCRβdel/TCRins] showing a significant reduction {Samples M^{P2}, M^{P3}, M^{P4} and M^{P6}}, while others {Samples M^{P1}, M^{P5} and M^{P7}} showed TRB-DJ1 transcript levels comparable to their littermate controls, C^P [TCRβdel/+] . The latter paternal mutants were useful for many of the analyses described in the later part of the present study as they served as controls in which the inserted TCRins was not organized into a functional insulator. The variability observed for paternal TCRins mutants in TRB-DJ1 transcript levels (Fig 14b) was also seen in case of TRB-DJ2 transcription (Fig. 15). At the endogenous *H19-Igf2* locus, hypermethylation of the *H19-ICR* is responsible for its inability to establish a functional insulator. Hence, to determine the basis for the variability in the transcription in paternal samples, methylation status of the inserted *H19-ICR* was analyzed. This aspect of the study is described in the next chapter.
Fig. 14. TRB-DJ1 germline transcription analysis for total thymocytes of paternal TCRγ δ inheritance samples. Quantitative RT-PCR was performed for paternal TCRγ δ mutants, TCRβδ+/−TCRγ δ {Mγ δ, cyan bars} and their littermate control mice, TCRβδ+/+ {Cγ δ, grey bars}. (a) Upper schematic diagram represents TCRβδ allele showing only forward primer’s (M387) position. The region corresponding to the reverse primer M386r is absent in TCRβδ allele. Lower schematic diagram of TCRγ δ allele with black arrows showing positions of primers, M387 and M386r for detection of TRB-DJ1 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCRγ δ allele. (b) Relative transcript levels of TRB-DJ1 normalized to Thy1.2 transcription. Each bar represents mean with ± SEM of measurements made in triplicates.
Fig. 15. TRB-DJ2 germline transcription analysis for total thymocytes of paternal TCR\textsubscript{Rins} inheritance samples. Quantitative RT-PCR was performed for TCR\textsubscript{Rins} mutants, TCR\textsubscript{Rins}/-TCR\textsubscript{Rins} \{\textit{M} \text{P}, cyan bars\} and their littermate control mice, TCR\textsubscript{Rins}/+ \{\textit{C} \text{P}, grey bars\}. (a) Lower schematic diagram represents TCR\beta\textsubscript{del} allele. The TCR\beta\textsubscript{del} allele does not possess region corresponding to both the primers. Lower Schematic diagram represents TCR\textsubscript{Rins} allele with black arrows showing positions of primers, M332 and M333\textsubscript{r} for detection of TRB-DJ2 transcripts. For the wild type allele, the positions of both the primers is the same as that of the TCR\textsubscript{Rins} allele. (c) Relative transcript levels of TRB-DJ2 normalized to Thy1.2 transcription. Each bar represents mean with ± SEM of measurements made in triplicates.
To conclude, the results presented in this chapter demonstrate that the inserted *H19-ICR* (in the TCRins mutant mice) acts as an efficient position-dependent enhancer blocking insulator in the heterologous context of *TCRβ* locus in case of its maternal inheritance. This proves that the *H19-ICR per se* is sufficient for establishment of a functional enhancer blocking insulator in the absence of any other cis-acting elements of the endogenous *H19-Igf2* locus which have been proposed to be important for establishment of a functional insulator by the *H19-ICR*. However, in case of paternal transmission of the inserted *H19-ICR*, variability was observed in TRB-DJ1 and TRB-DJ2 transcript levels. Thus, in the heterologous context of *TCRβ* locus, some paternal TCRins mutants showed an unexpected establishment of a functional insulator.