Brief report

The mouse Runx1 +23 hematopoietic stem cell enhancer confers hematopoietic specificity to both Runx1 promoters

Thomas Bee,1 Emma L.K. Ashley,1 Sorrel R.B. Bickley,1 Andrew Jarratt,1 Pik-Shan Li,1 Jackie Sloane-Stanley,1 Berthold Göttnens,2 and Marella F.T.R. de Bruijn1

1Medical Research Council Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford University, Oxford; and
2Cambridge University Department of Haematology, Cambridge Institute for Medical Research, Cambridge, United Kingdom

The transcription factor Runx1 plays a pivotal role in hematopoietic stem cell (HSC) emergence, and studies into its transcriptional regulation should give insight into the critical steps of HSC specification. Recently, we identified the Runx1 +23 enhancer that targets reporter gene expression to the first emerging HSCs of the mouse embryo when linked to the heterologous hsp68 promoter. Endogenous Runx1 is transcribed from 2 alternative promoters, P1 and P2. Here, we examined the in vivo cis-regulatory potential of these alternative promoters and asked whether they act with and contribute to the spatiotemporal specific expression of the Runx1 +23 enhancer. Our results firmly establish that, in contrast to zebrafish runx1, mouse Runx1 promoter sequences do not confer any hematopoietic specificity in transgenic embryos. Yet, both mouse promoters act with the +23 enhancer to drive reporter gene expression to sites of HSC emergence and colonization, in a +23-specific pattern. (Blood. 2009;113:5121-5124)

Introduction

The transcription factor RUNX1 is a critical regulator of definitive hematopoiesis, and genomic aberrations of the gene encoding RUNX1 are frequently found in human acute leukemia.1 In the mouse, Runx1 null mutations result in the absence of functional hematopoietic stem cells (HSCs) and definitive progenitors, leading to embryonic lethality.2-6 During development, Runx1 is first expressed in the emerging hematopoietic system, including definitive HSCs.7,8 Its highly regulated spatiotemporal expression pattern and pivotal role in HSC emergence prompted us to study its transcriptional regulation, to obtain insight into the molecular mechanisms underlying de novo HSC generation. We recently identified the Runx1 +23 hematopoietic enhancer, located 23.5 kb downstream of the ATG in exon 1.9 We showed that this +23 enhancer targets reporter gene expression, from a heterologous hsp68 core promoter, to the emerging HSCs and putative HSC-fated cells in the mouse embryo, and acts directly downstream of Gata2, SCL, and Ets transcription factors. Whether the +23 enhancer is equally active with the endogenous Runx1 promoters has not been assessed.

Runx1 is transcribed from 2 alternative promoters (Figure 1A), a distal P1 and proximal P2, with the P1 being specific to vertebrates.10,11 Both the P1 and P2 promoters were reported to be transcriptionally active in the emerging hematopoietic system of the mouse embryo, at the stages of yolk sac (YS), aorta-gonad-mesonephros (AGM), and fetal liver (FL) hematopoiesis, with P1-derived transcripts particularly prevalent among enriched FL HSCs.12,14,15 The P2 promoter was shown to be active in HSC-fated cells16 and to be critically required for FL hematopoiesis.17 In vitro transfection assays suggested that neither P1 nor P2 RUNX1 promoter elements harbored tissue-specific cis-regulatory elements.10 However, in vivo mouse promoter assays have not been reported, and it is therefore not clear to what extent cis-elements elsewhere in the locus are required to mediate the activity of the 2 Runx1 promoters.

Methods

Cloning

Genomic fragments spanning the mouse Runx1 P1 and P2 promoters were generated by polymerase chain reaction (PCR) using the primers listed (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Fragments were cloned upstream of the luciferase gene in pGL3 vectors (Promega, Southampton, United Kingdom) and upstream of the LacZ reporter, with or without the Runx1 +23 enhancer.9 Cloning strategy and maps are available on request. Mutagenesis of conserved Runx motifs in the P1 promoter was performed as described,9 using the primers listed in Table S1.

Promoter assays

Luciferase assays were performed in the 416B myeloid progenitor cell line.9 Mouse F0 transgenic embryos were generated and analyzed as described.3 All mouse work was in accordance with United Kingdom Home Office regulations.

Chromatin immunoprecipitation

Tissues were harvested and chromatin immunoprecipitation (ChIP) performed as described.9

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Results and discussion

To establish whether mouse Runx1 promoter sequences have any cis-regulatory activity of their own, mouse P1 (526 bp) and P2 (205 bp) promoter fragments (Figure 1B) were cloned upstream of the LacZ reporter gene for analysis in F0 transgenic embryos. These elements spanned the areas of multispecies conservation around the mouse homologs of core promoter sequences defined in the human RUNX1 promoters and were confirmed to be active in vivo, with the P2 marking the zebrafish equivalents of mouse P1 and P2 promoter constructs in transiently transfected 416B myeloid progenitor cells. Constructs with the SV40 core promoter were used as controls. Data are the mean plus or minus SD of more than or equal to 5 independent transfections, using more than or equal to 2 separately prepared batches of test plasmids. (D) In vivo analysis of mouse P1 and P2 promoter fragments in F0 transgenic mouse embryos. Embryos were analyzed for LacZ expression as whole mounts and after cryosectioning as before. 

No reproducible Runx1-specific Xgal staining could be observed in F0 transgenic mouse embryos carrying the P1 LacZ (n = 10) or P2 LacZ (n = 20) transgenes. Nonspecific staining, or no staining, is presumably the result of random integration of the constructs at or near endogenous enhancers, or in heterochromatin, respectively. (E) Real-time PCR analysis of ChIP for SCL and Gata2 in mouse (CBAxC57BL/6)/F1 FL cells and 416B cells, respectively. ChIP for SCL shows strong enrichment for the P2 (+23) and to a lesser extent the P1, but not the P2. Both the +23 and the P1 harbor a mouse-frog conserved Gata motif, whereas the putative Gata motif in the P2 is only conserved between mouse, human, and dog (not shown). For SCL, data are the mean plus or minus SD of 2 independent ChIP experiments with 2 real-time PCR assays per ChIP; for Gata2 one representative experiment is shown. No Ab indicates no antibody control. Runx1 +23 enhancer and P1 and P2 promoter primers and probes used for real-time PCR are listed in Table S2.

Figure 1. In vitro and in vivo functional analysis of mouse Runx1 promoter fragments. (A) Schematic of mouse Runx1 locus showing the location of the P1 and P2 promoters, the +23 hematopoietic enhancer (black arrow) and exons (light blue indicates untranslated region [UTR]; dark blue, coding sequences). (B) Vista plots showing percentage identity in pairwise alignments of the mouse Runx1 P1 and P2 promoter fragments used in this study and their corresponding human (Homo sapiens), dog (Canis familiaris), opossum (Monodelphis domestica), chicken (Gallus gallus), and frog (Xenopus tropicalis) sequences. A 6-way RankVista alignment indicates the extent of multispecies conservation and the likelihood (–log10[P value]) this conservation arose by chance. Genomic sequences and alignments as in Nottingham et al. Pink denotes areas of more than 70% noncoding sequence conservation more than 100 bp; light blue, conserved UTRs. (C) Luciferase activity of mouse P1 and P2 promoter constructs in transiently transfected 416B myeloid progenitor cells. Constructs with the SV40 core promoter were used as controls. Data are the mean plus or minus SD of more than or equal to 2 independent transfections, using more than or equal to 2 separately prepared batches of test plasmids. (D) In vivo analysis of mouse P1 and P2 promoter fragments in F0 transgenic mouse embryos. Embryos were analyzed for LacZ expression as whole mounts and after cryosectioning as before. 

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the activity of the resulting constructs analyzed in F0 transgenic mouse embryos. In extensive analysis of both P1 LacZm +23 (n = 58) and P2 LacZ +23 (n = 44) F0 transgenic embryos, Xgal staining was reproducibly observed at sites of developmental hematopoiesis (Figure 2A,B), in proportions and a pattern similar to that seen in hsp68 LacZ +23 F0 transgenics, ie, in the blood islands of the E8 YS, in hematopoietic clusters and a few endothelial/mesenchymal cells of the E10 dorsal aorta and vitelline and umbilical arteries, and in E12 FL hematopoietic cells, as previously observed in hsp68 LacZ +23 F0 transgenic embryos.9 Non-specific staining, or no staining, is presumably the result of random integration of the constructs at or near endogenous enhancers, or in heterochromatin, respectively. (B) Representative photographs of the +23-specific Xgal staining in P1 LacZ +23 (Bi-iii) and P2 LacZ +23 (Biv-vi) F0 transgenic embryos. (Bi,v) Staining in E8 YS blood islands. (Biv,v) Staining in emerging clusters and few endothelial cells in the E10 dorsal aorta, (Biii,v) Staining in E12 FL hematopoietic cells. ▶ points at examples of Xgal-stained cells. Figure S3 shows negative control. (C) Schematic of the Runx1 P1 promoter region showing the location and sequence of the deeply conserved pair of Runx motifs. Basepairs critical for DNA binding were identified as shown. Pink denotes the area of noncoding sequence conservation spanning the promoter; light blue, the UTR; dark blue, the coding sequence of exon 1. (D) Summary of mutated P1 promoter activity in P1mut LacZ +23 F0 transgenic mouse embryos. The lack of expression in the E8 P1mut LacZ +23 F0 transgenic embryos is probably a frequency issue (only 2 of 16 P1 LacZ +23 embryos [Figure 2A] were Xgal+). (E) Mutation of the deeply conserved P1 Runx motifs does not alter the activity of the P1mut LacZ +23 construct in vivo. Representative Xgal staining in P1mut LacZ +23 F0 transgenic mouse embryos in E10 YS (Ei), in emerging hematopoietic clusters of the E10 vitelline artery (Eii) and dorsal aorta (Eiii), and in E12 FL cells (Eiv). ▶ points at examples of Xgal-stained cells. Photographs were taken using a Nikon Eclipse E600 microscope equipped with a 20× Nomarski objective and a Nikon DXM 1200c Digital Camera (Nikon, Tokyo, Japan) and processed using Adobe Photoshop (Adobe Systems Europe, Uxbridge, United Kingdom). Scale bar represents 100 μm.

To begin to address the transcriptional regulation of the Runx1 promoters, we examined the relevance of 2 deeply conserved Runx motifs for P1 activity (Figure 2C). These Runx motifs are conserved down to frog22 and are present in the P1 promoters of all 3 Runx genes,13 suggesting a deeply conserved role in positive or negative auto- and/or cross-regulation.23 Indeed, the Runx1 P1 was demonstrated to be subject to a positive auto-regulatory and a negative cross-regulatory loop in myeloid and B-cell lines, respectively.22,24 In mouse AGM hematopoiesis and during ES cell differentiation, the Runx1 P1 was suggested to be subject to auto-regulation.7,14 Thus, we mutated the pair of deeply conserved Runx motifs and analyzed the activity of the resulting P1mut LacZ +23 construct in the developing hematopoietic system of F0 transgenic embryos. Surprisingly, mutation of these motifs did not significantly alter in vivo transgene activity, compared with the P1LacZ +23 construct, in the E10 YS, in the hematopoietic
clusters of the dorsal aorta and vitelline/umbilical arteries, or in the E12 FL (Figure 2D,E). We concluded that the pair of conserved Runx motifs is not required for P1 promoter activity during mouse developmental hematopoiesis. Whether they play a role in other organ systems or in interactions with other Runx1 cis-elements remains to be established.

In conclusion, we firmly established that the Runx1 +23 HSC enhancer confers its hematopoietic specificity to both Runx1 P1 and P2 promoter fragments in vivo. Furthermore, we demonstrated that there is no role for the pair of conserved Runx motifs in a long-proposed Runx1 auto-regulatory loop in developmental hematopoiesis. Finally, the observation that the P1 and P2 promoter fragments on their own lacked any reproducible Runx1-specific activity in vivo indicates that both the specific hematopoietic and nonhematopoietic expression of Runx1 critically relies on enhancer elements elsewhere in the locus. Future identification of cell- and/or stage-specific Runx1 hematopoietic enhancers will provide the basis for building gene-regulatory networks underlying HSC specification and differentiation.

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Authorship

Contribution: T.B. performed the research, analyzed data, and drafted the paper; E.L.K.A., S.R.B.B., and A.J. performed research and analyzed data; P.-S.L. and J.S.-S. generated transgenics; B.G. performed the genomics analyses and critically commented on the paper; and M.F.T.R.d.B. designed the study, analyzed data, and wrote the paper.

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Correspondence: Marella F. T. R. de Bruijn, Medical Research Council Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, OX3 9DS, United Kingdom; e-mail: marella.debruijn@imm.ox.ac.uk.

References

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