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Transcriptional Regulation and Spatial Organisation of the Human *AML1/RUNX1* Gene

Elena N. Markova, Omar L. Kantidze,* and Sergey V. Razin*

Laboratory of Structural and Functional Organization of Chromosomes, Institute of Gene Biology RAS, Moscow, Russia

ABSTRACT

The transcription factor RUNX1 is a key regulator of haematopoiesis in vertebrates. In humans, the 260-kb long gene coding for this transcription factor is located on chromosome 21. This gene is transcribed from two alternative promoters that are commonly referred to as the distal and the proximal promoters. In model experiments, these two promoters were found to be active in cells of different lineages, although *RUNX1* is preferentially expressed in haematopoietic cells. In the present study, we attempted to identify the regulatory elements that could guide tissue-specific expression of the *RUNX1* gene. Two such regulatory elements were found within the *RUNX1* gene. One of these elements, located within intron 1, is a haematopoietic-specific enhancer. The second regulatory element, located within intron 5.2, contributes to the formation of an active chromatin hub, which integrates the above-mentioned enhancer and the P1 and P2 promoters. J. Cell. Biochem. 112: 1997–2005, 2011.

KEY WORDS: CHROMATIN; ENHANCERS; ALTERNATIVE PROMOTERS; TRANSCRIPTION CONTROL; CHROMOSOME CONFORMATION CAPTURE; CHROMATIN HUB

The human protein RUNX1 (also known as AML1) belongs to a family of evolutionarily conserved core-binding transcription factors (CBFs). These transcription factors consist of two proteins: a variable DNA-binding subunit, CBFα, and an invariable non-DNA-binding subunit, CBFβ. The CBFα subunit in diverse cell types can be presented by one of the three proteins: RUNX1, RUNX2 or RUNX3 [Westendorf and Hiebert, 1999]. All three RUNX proteins are key regulators of lineage-specific gene expression in major developmental pathways. The RUNX1 protein is a crucial regulator of definitive haematopoiesis [Otto et al., 2003]. The structure of this protein was elucidated several years ago. It consists of two well-defined domains: a runt homology domain (RHD) that is responsible for recognising DNA at a special site TGT/CGGT and a transactivation domain (TAD) [Bartfeld et al., 2002; Bernardin and Friedman, 2002]. The participation of human RUNX1 protein in a complicated network of pathways, guiding cellular differentiation during haematopoiesis, has been the subject of many recent studies. Generally, this transcription factor serves as a platform for the proper assembly of a vast number of different transcription factors at the promoter regions of the target genes [Kim et al., 1999; Zhang et al., 2008; Zhao et al., 2008; Bakshi et al., 2010; Lichtinger et al., 2010].

Much is known about the structure of the gene coding for the RUNX1 protein. The human *RUNX1* gene is located on chromosome 21q22.12 and spans 260 kb. Its transcription can start from one of the two promoters (P1 or P2), which are located 160 kb apart [Ghozi et al., 1996]. RUNX1 is encoded by 12 exons that can be alternatively spliced. Exons 2, 3 and 4 code for the RHD, and exon 6 codes for the TAD (Fig. 1). A number of different isoforms of the RUNX1 protein, from the full-length protein to the truncated forms that contain only the DNA-binding domain, can be produced as a result of the transcription from alternative promoters, which is followed by alternative splicing [Levanon et al., 2001].

The *RUNX1* gene is notorious for its frequent participation in chromosomal translocations, which usually lead to acute forms of leukaemia [Bystritskiy and Razin, 2004].

Although the translocation events resulting in a fusion of *RUNX1* gene with different partners have been intensively studied [Elagib

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*Correspondence to: Omar L. Kantidze and Sergey V.Razin, Institute of Gene Biology RAS, 34/5 Vavilova Street, 119334 Moscow, Russia. E-mail: omar.kantidze@genebiology.ru; sergey.v.razin@usa.net

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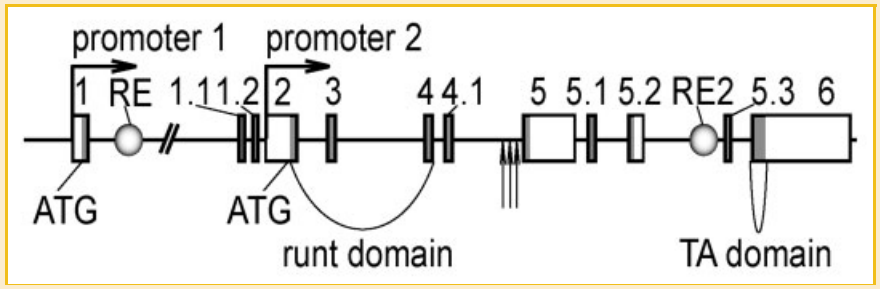

Fig. 1. Genomic organisation of the human *RUNX1* gene [Levanon et al., 2001]. Square boxes represent exons. The white filling inside the boxes indicates UTRs. The round beads represent the putative enhancer elements under study. The three vertical arrows inside the 4.1 intron mark the breakpoint cluster regions that are characteristic of the t(8;21) and t(3;21) chromosomal translocations.

and Goldfarb, 2007; Ito, 2008; Dowdy et al., 2010], the mechanism directing the tissue-specific expression of the non-rearranged human *RUNX1* gene remains unclear. This gene was shown to be highly active in the haematopoietic system but not in other tissues (brain, lung, ovaries and testis) [Levanon et al., 1994, 1996; Nucifora and Rowley, 1995]. However, neither of the two *RUNX1* promoters possessed any tissue specificity as assessed by using transfections of constructs with a reporter gene driven by either the P1 or P2 promoters [Ghozi et al., 1996]. At the same time, both promoters responded to the activation by a lymphoid-specific mouse TCRβ enhancer [Ghozi et al., 1996]. It is reasonable to suggest that there should be haematopoietic cell-specific enhancers that control the tissue-specific expression of the human *RUNX1* gene. In the present study, we attempted to identify such regulatory elements. Based on the analysis of interspecies DNA sequence homology and on the analysis of the distribution of lymphoid cell-specific DNase I hypersensitive sites (DHSs) in the *RUNX1* locus, we identified two DNA fragments that may harbour regulatory sequences. We tested the above-mentioned DNA fragments in a transient transfection assay and demonstrated that one of the fragments possessed a haematopoietic cell-specific enhancer activity for both promoters of the *RUNX1* gene. Using the chromosome conformation capture (3C) analysis, we demonstrated that both the above-mentioned DNA fragments and the P1 and P2 promoters were assembled into a common chromatin hub in lymphoid and erythroid cells.

MATERIALS AND METHODS

CELL CULTURE

The human acute T-cell leukaemia cell line Jurkat (ATCC) and chronic myelogenous leukaemia cell line K562 (ATCC) were grown in RPMI-1640 medium, supplemented with 10% foetal bovine serum at 37°C in a 5% CO_2 atmosphere. The human embryonic kidney cell line HEK293 (ATCC), was grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% foetal bovine serum at 37°C in 5% CO_2 atmosphere.

GENE EXPRESSION ANALYSIS

RNA was extracted from cells and tissues by using Trizol reagent (Invitrogen). All RNA samples were further treated with DNase I (Fermentas) to remove residual DNA. RNA (1 μg) was reverse transcribed in a total volume of 20 μl for 1 h at 42°C, using 0.4 μg random hexamer primers and 200 U reverse transcriptase (Fermentas) in the presence of 20 U of ribonuclease inhibitor (Fermentas). The cDNA obtained was analysed by quantitative PCR using the CFX96 real-time PCR detection system (Bio-Rad). A PCR mixture in a volume of 20 μl contained 50 mM Tris–HCl (pH 8.6), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Tween-20, 0.5 μM of each primer, 0.25 μM of TaqMan probe (5′-FAM dye, inside -BHQ-1 quencher), 0.2 mM of each dNTP, 0.75 U of Hot Start Taq Polymerase (Sibenzyme) and 25 ng of cDNA template. The PCR reactions were performed as follows: initial denaturation for 5 min at 94°C; 45 cycles of 15 s at 94°C; 60 s at 60°C; the plate was then read. Each PCR was performed in quadruplicate, and the corresponding results were averaged. The sequences of the primers and TaqMan probes find in Tables I and II accordingly.

LUCIFERASE REPORTER CONSTRUCTS PREPARATION

Promoters P1 and P2 and DNA fragments containing putative enhancers RE1 and RE2 were obtained using PCR amplification of human genomic DNA with specially designed primers. Lymphoid-specific enhancer TCRβ from *Mus musculus* was obtained by PCR amplification of mouse genomic DNA with the corresponding primers. The sequences of the primers find in Table III. The promoters were cloned upstream from the *Luciferase* gene in the pGL3-Basic vector (Promega). Fragments RE1, RE2 and TCRβ were cloned into the same vectors downstream from the *Luciferase* gene in two possible orientations: direct genomic and reverse genomic. TCRβ was cloned only in the direct orientation. All manipulations with recombinant DNA vectors were performed according to standard protocols [Maniatis et al., 1989]. The structure of the final genetic constructs was confirmed by sequencing and restriction analysis.

TABLE I. Primers Used for *RUNX1* Expression Analysis

RUNX1_control_direct	5'-TTAGTTGTCAGCAGGTAAAGCC-3
RUNX1_control_reverse	5'-ATAGAAGGGGAACCAGGAGG-3'
RUNX1_exon1_direct	5'-CCGCCTTCAGAAGAGGGT-3'
RUNX1_exon1_reverse	5'-TGAAGCACTGTGGGTACGAA-3'
RUNX1_exon2_direct	5'-AGCCCAGGCAAGATGAGC-3'
RUNX1_exon2_reverse	5'-GTAGGCAGCACGGAGCAG-3'

TABLE II. TaqMan Probes Used for *RUNX1* Expression Analysis

RUNX1_control_taqman	5'-(FAM)CAGGCATGT(BHQ-1)CTTTCTAAGGTAGAGGACG-3'
RUNX1_exon1_taqman	5'-(FAM)TGGCTTCAGACAGCAT(BHQ-1)ATTTGAGTCATTT-3'
RUNX1_exon2_taqman	5'-(FAM)CAAGCT(BHQ-1)GAGGAGCGGCGACC-3'

TRANSIENT TRANSFECTION EXPERIMENTS

Transfection of the luciferase constructs into the Jurkat, K562 and HEK293 cell lines was performed using TurboFect® (Fermentas) as follows: 2×10^6 of Jurkat or K562 suspension cells (7×10^5 adherent HEK293 cells) were treated with 6 μl of reagent (4 μl for adherent cells) and DNA solution containing 4 μg (2 μg for adherent cells) of each test construct and 0.4 μg (0.2 μg for adherent cells) of pRL-CMV (Promega). The relative luciferase activity was determined after 48 h by using the Dual-Luciferase® Reporter Assay System (Promega), according to the manufacturer's instructions. The ratio of firefly to renilla luciferase activity was used to correct for the transfection efficiency.

CHROMOSOME CONFORMATION CAPTURE ANALYSIS

3C analysis was performed as previously described [Splinter et al., 2004; Gavrilov and Razin, 2008]. A random-ligation control was generated by using DNA from a bacterial artificial chromosome that contained the human *RUNX1* gene and the flanking areas (*Homo sapiens* BAC clones RP11-77G18 and RP11-177L11, CHORI BACPAC Resources Centre). The ligation products were analysed using real-time PCR with TaqMan probes. The primers and TaqMan probes for PCR analysis were designed using the DNA sequence of the human *RUNX1* gene (GenBank ID NG_011402). The sequences of the primers and the TaqMan probes find in Tables IV and V accordingly. An internal standard was used to account for the differences in the efficiency of crosslinking/restriction/ligation and in the quantity of DNA in the 3C templates obtained from cells of different types [Splinter et al., 2004]. The *ERCC3* locus has been reported to adopt the same spatial conformation in different tissues [de Laat and Grosveld, 2003; Palstra et al., 2003; Vernimmen et al., 2007]. Thus, all 3C results were corrected by data from *ERCC3* analysis, controlling for changes in nuclear size, chromatin density and crosslinking efficiency.

RESULTS

THE ACTIVITY OF PROMOTERS P1 AND P2 VARIES GREATLY IN DIFFERENT CELL LINEAGES

To gain further insight into the regulation of human *RUNX1* gene expression, we analysed the expression profile of this gene in cultured human cells of different lineages: HEK293 epithelial cells, Jurkat lymphoid cells and K562 erythroid cells. In the first set of experiments, we attempted to estimate the relative activity of the *RUNX1* gene P1 and P2 promoters in these cell lines. For this purpose, we compared the representation of exonic regions located between P1 and P2 (i.e., transcribed from P1) and downstream to P2 (i.e., transcribed from both P1 and P2) in the total RNA. The positions of test-amplicons are shown in Figure 2A. The test-amplicon located upstream to P1 was considered as a negative control. The total RNA was isolated from the above-mentioned cultured cells, and the resulting cDNA was analysed using real-time PCR with primers and probes described in the Material and Methods Section. The row data were normalised to the experimentally determined level of one of the exons of the housekeeping *GAPDH* gene. The results of the analysis are shown in Figure 2B. In the HEK293 epithelial cells, promoter P1 was silent, and promoter P2 displayed very low activity. In lymphoid cells, both promoters were almost equally active. In erythroid cells, promoter P2 was 10-fold more active than promoter P1.

TABLE III. Primers Used for the Preparation of Genetic Constructs

T7	5'-TAATACGACTCACTATAGGG-3'	
SP6	5'-ATTTAGGTGACACTATAGAA-3'	
RUNX1_P1_direct	5'-TAATGCTAGCGCCAGCGTTGAATTA-3'	*Nhe*I
RUNX1_P1_reverse	5'-TAATCTCGAGAGGCCCAAAGAAGTT-3'	*Xho*I
RUNX1_P2_direct	5'-TAATGCTAGCCGGGCTGCGTACAGT-3'	*Nhe*I
RUNX1_P2_reverse	5'-TAATCTCGAGCCGGAAAGAAGTGCC-3'	*Xho*I
RUNX1_RE1_direct	5'-TAATGTCGACAGGGTGCGGGCTTCA-3'	*Sal*I
RUNX1_RE1_reverse	5'-TAATGTCGACCGGGGACTTGTTGGT-3'	*Sal*I
RUNX1_RE2_direct	5'-TAATGTCGACTAGCCCTTTCCCAGA-3'	*Sal*I
RUNX1_RE2_reverse	5'-TAATGTCGACCACCACTCCCTGCTT-3'	*Sal*I
TCR_control_direct	5'-TAATGTCGACAGGATCTGCTAAAAC-3'	*Sal*I
TCR_control_reverse	5'-TAATGTCGACGTAAATGTCAAACCA-3'	*Sal*I

IDENTIFICATION OF A HAEMATOPOIETIC CELL-SPECIFIC ENHANCER IN THE FIRST INTRON OF *RUNX1* GENE

Our next aim was to choose those genomic elements that could possibly possess tissue-specific enhancer activity towards one of the promoters. We selected two regions of interest located within the *RUNX1* gene which further are referred to as RE1 (regulatory element 1) and RE2 (regulatory element 2) (Fig. 1). RE1 fragment 500 bp in size is located inside the intron 1. The corresponding element called '+23 (1-531)' was previously identified as a conserved non-coding element in vertebrates bearing enhancer activity in mouse myeloid cells [Nottingham et al., 2007]. RE2 is a region 400 bp in size located 12.5 kb downstream from exon 5.2. It contains three lymphoid-specific DHSs which coincide with three topoisomerase II cleavage sites [Zhang et al., 2002]. The occurrence of DHSs within a DNA fragment usually reflects the presence of protein-binding sites [Boyes and Felsenfeld, 1996] and therefore indicates the presence of regulatory elements. To test the putative

TABLE IV. Primers Used for 3C Analysis

RUNX1_P1_left	5'-GAGATGTGTCCTGTGTGGGC-3'
RUNX1_P1	5'-ACTTAGTTATGCTGTTGGAGTGTTC-3'
RUNX1_P1_right	5'-CCCACACAGGAAACTCAAGC-3'
RUNX1_RE1_left	5'-ATTTGCCAGTATTCTTTCCCTT-3'
RUNX1_RE1	5'-GTTTATTGGCTAAACAAGTAAATCC-3'
RUNX1_RE1_right	5'-GCGTGTGTAAAATGAGCCTG-3'
RUNX1_P2_left	5'-AGGCTGTTTCTGCTGATTCC-3'
RUNX1_P2	5'-CTTTCCTGCTAGAGGAGGGG-3'
RUNX1_P2_right	5'-GGGTCATTTCCTGTTCGTTT-3'
RUNX1_RE2_left_2	5'-CAGGGCTCAAATCCTTCAAA-3'
RUNX1_RE2_left_1	5'-CACCTCAATGGAGTGTTCTTCA-3'
RUNX1_RE2	5'-AAAAGAAAAGGACTCCAGGGTG-3'
RUNX1_RE2_right_1	5'-CTCTTCTGCTTGCTTGAGGTCT-3'
RUNX1_RE2_right_2	5'-CTGACTGTAAGTGCCTGTGGG-3'
ERCC3_1	5'-CCAGTTGTTAGGTTGGGAAAG-3'
ERCC3_2	5'-ACAGAAGCGGTGAGGTGAGTT-3'

TABLE V. TaqMan Probes Used for 3C Analysis

RUNX1_P1_taqman	5'-(FAM)CTTGACAAAGT(BHQ-1)TCTCACGCACCGAC-3'
RUNX1_P2_taqman	5'-(FAM)TGGGGATGAT(BHQ-1)CAGGGGTGAAAAGTG-3
RUNX1_RE2_taqman	5'-(FAM)TGAATCAAGAAT(BHQ-1)GCTGGTTCCTCACA-3'
ERCC3_taqman	5'-(FAM)CAGTTGGGT(BHQ-1)GGGCTACACAGCAGTC-3'

enhancer activity of RE1 and RE2, we made a number of genetic constructs with a pGL3-Basic vector, which contains the firefly *Luciferase* gene. We prepared two identical sets of constructs: one containing promoter P1 and the other one with promoter P2. As promoter P1 we used a DNA fragment corresponding to the distal *RUNX1* promoter region −12 to −370 and as promoter P2 we took a proximal *RUNX1* promoter region −190 to +111 identified previously by Ghozi et al. [1996]. The promoters were cloned upstream from the *Luciferase* gene and candidate enhancer elements—downstream of the gene in direct genomic orientation and reverse genomic orientation relative to the corresponding promoter. In each set of experiments, we used a well-known lymphoid-specific TCRβ enhancer from *M. musculus* [Krimpenfort et al., 1988] as a positive control. As a universal control for both sets of genetic constructs, we used pGL3-Control vector in which the expression of the *Luciferase* gene is controlled by the SV40 promoter and SV40 enhancer. The resulting genetic constructs were transfected into HEK293, Jurkat and K562 cells. After a 48 h incubation, the luciferase activity in the cell extracts was determined. The results are presented in Figure 3. The activity observed for the pGL3-control vector was taken as 100%. In epithelial cells, both promoters were active, although the activity of P1 was about three times lower than the activity of P2. TCRβ, RE1 and RE2 did not produce a significant increase in the activity of the P1 and P2 promoters. RE1 displayed low silencing activity towards the P1 and P2 promoters, as shown by a 30–40% decrease in luciferase activity. In the K562 cells, the P1 and P2 promoters showed comparable basal activity. In this cell line, TCRβ enhancer, RE1 and RE2 did not display any enhancer activity towards promoter P1. However, RE1 (when present in a direct orientation) stimulated nearly twice the activity of P2 promoter as compared to RE2, which did not show either enhancing or silencing activity towards the P2 promoter. In the Jurkat lymphoid cells, the basal activity of the P1 and P2 promoters was about the same, and it was significantly (especially in the case of P2) stimulated by both the TCRβ enhancer and RE1. The enhancing activity of RE1 in the Jurkat cells did not depend on the orientation. As in the HEK293 and K562 cells, RE2 did not influence the activity of either P1 or P2 in the Jurkat cells.

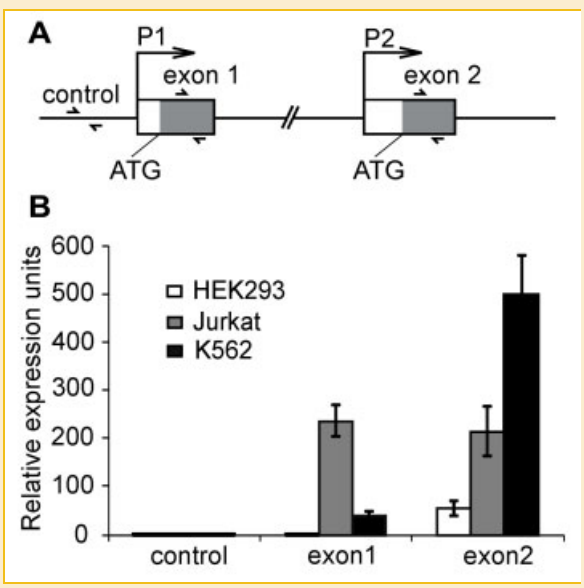

Fig. 2. The human *RUNX1* gene expression profiles in cells of different lineages. A: A scheme showing the position of the primers used for the analysis. B: Relative abundance of exon 1 (which is present only in P1 transcripts) and exon 2 (which is present in both P1 and P2 transcripts) in total RNA isolated from HEK293 (white columns), Jurkat (grey columns) and K562 (black columns) cells. All of the values are normalised to the level of the *GAPDH* exonic sequence. The error bars represent the standard error in measurement (SEM) for four independent experiments.

RUNX1 PROMOTERS DIRECTLY INTERACT WITH EACH OTHER AND WITH RE1 AND RE2 IN LYMPHOID AND ERYTHROID CELLS

Enhancers can be situated very far from the corresponding promoters on the chromosome. However, inside the nucleus, the promoters and the enhancers can be brought into close proximity through DNA looping, which is mediated by protein complexes [Tolhuis et al., 2002; Splinter et al., 2006; Palstra et al., 2008]. In the *RUNX1* gene, RE1 and RE2 are located far from promoters P1 and P2. Thus, we were curious to determine if they are situated close to each other within the nucleus. The 3C procedure was employed to address this question [Dekker et al., 2002]. For this method, chromatin is fixed with formaldehyde in vivo to crosslink interacting sites, digested with a restriction enzyme and ligated at a low DNA concentration so that ligation between crosslinked fragments is favoured over ligation between random fragments. The ligation products are then analysed and quantified by real-time PCR [Hagege et al., 2007; Gavrilov and Razin, 2008]. We performed 3C analysis on the HEK293, Jurkat and K562 cell lines. We chose the *Hin*d III restriction endonuclease to digest the DNA after cross-linking because it cuts the *RUNX1* gene locus into fragments ranging from 3 to 10 kb in size, and all four elements (promoter P1, promoter P2, RE1 and RE2) are located in different restriction fragments. The *RUNX1* gene is quite long, so we chose only certain fragments for analysis. We selected the fragments that contained P1 and P2 promoters and the fragments that contained RE1 and RE2 as well as the flanking regions. Primers for real-time PCR analysis were designed to anneal at the right ends of the selected restriction fragments and face outwards. In this way head-to-head ligation products were analysed. One primer (different in different sets of experiments) was used as an anchor primer to carry out PCR with all other primers. Each anchor primer was linked to a TaqMan which was placed between the primer and downstream end of the restriction fragment. We used three anchor primers—one primer for the fragment bearing promoter P1, one for the fragment bearing promoter P2 and the third one for the restriction fragment harbouring RE2 element. Unidirectionality of the primers eliminated

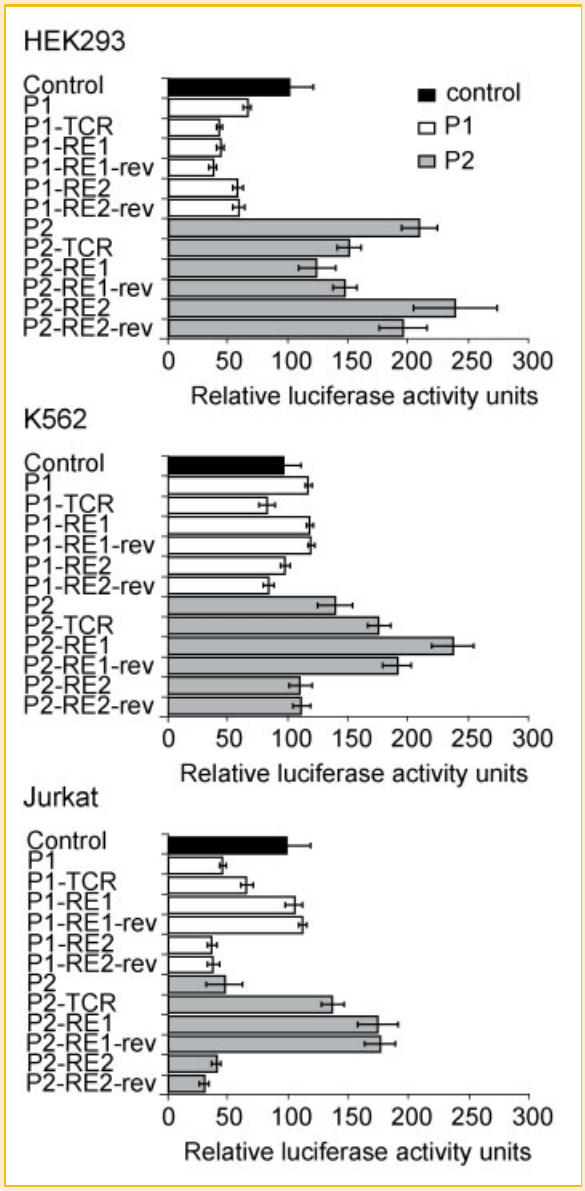

Fig. 3. Testing of RE1 and RE2 genomic fragments for enhancer activity in human Jurkat, K562 and HEK293 cells. The names of the constructs are shown on the left side of the diagrams. The diagrams show the normalised luciferase activity. The activity observed for the pGL3-control vector (black columns) was arbitrarily considered as '100', and the other data were normalised accordingly. The white and grey columns show the results for the series of constructs containing correspondingly the P1 promoter and the P2 promoter. The error bars represent SEM for three independent experiments.

the possibility of generating PCR products in case of partial digestion and subsequent ligation through circularization. The analysed restriction fragments and the positions of the corresponding primers and probes used for real-time PCR are shown in Figure 4A. To compare three different cell lines, we needed an internal control region for normalisation. The human *ERCC3* gene was chosen as this internal control. *ERCC3* locus has been reported to adopt the same spatial conformation in different tissues and therefore it is commonly used as internal control in 3C experiments worldwide [de Laat and Grosveld, 2003; Palstra et al., 2003; Vernimmen et al., 2007] (see the Materials and Methods Section for details). The final results are shown in Figure 4. The relative crosslinking frequency of two specific restriction fragments designated as a single column on the diagram was measured by the amount of corresponding ligation product detected through real-time PCR analysis. It is proportional to the frequency with which these two genomic sites interact. Thus, in the Jurkat and K562 cells, promoter P1 appears to interact with RE1, P2 and RE2 (Fig. 4B). In epithelial cells, P1 interacts only with the RE2 fragment. We wanted to determine with which elements P2 interacts. Therefore, we put the anchor on promoter P2. In the Jurkat and K562 cells, P2 interacts with promoter P1, RE1 and RE2 (Fig. 4C). However, in the HEK293 cells, P2 interacts only with RE2. To verify the observed interactions, we carried out 3C analysis with the anchor placed on the RE2 fragment. In Jurkat and K562 cells, we observed similar interactions between all four elements—P1, P2, RE1 and RE2 (Fig. 4D). In the HEK293 cells, RE2 also interacted with P2, P1 and RE1, but the frequency of the RE2-P2 interaction was nearly threefold greater than the frequency of the RE2-P1 and RE2-RE1 interactions.

DISCUSSION

The human *RUNX1* gene has been studied in many laboratories. However, these studies are primarily focused on pathologies ranging from point mutations to severe devastations such as chromosomal translocations [Osato et al., 1999; Hromas et al., 2000; Speck and Gilliland, 2002; Roumier et al., 2003; Paulsson et al., 2006]. The fundamental mechanism underlying the regulation of *RUNX1* expression in normal cells has not been well studied. The direct transcription regulation of the expression of this gene in humans remains unclear.

The first challenge towards understanding *RUNX1* gene expression is the number of promoters. Two promoters are located 160 kb apart from each other (Fig. 1) and are capable of directing transcription for a reporter gene in both haematopoietic and non-haematopoietic cell lines [Ghozi et al., 1996]. In developmental haematopoiesis, murine *Runx1* is transcribed from both P1 and P2. The results of recent studies emphasise the non-redundant functions of P1 and P2 in the onset of haematopoiesis. In adult haematopoietic cells, P1 acquires the role of being the main *Runx1* promoter [Bee et al., 2009, 2010; Sroczynska et al., 2009]. Our analysis has demonstrated that the activity of both human *RUNX1* gene promoters is similar in lymphoid Jurkat cells. However, P2 was shown to be several times more active in epithelial (HEK 293) and erythroid progenitor (K562) cells. Although K562 cells are human erythroleukaemic cell line of adult origin, they seem to acquire some features of primitive erythropoietic cells because they express embryonic β-type globin gene ε upon differentiation [Enver et al., 1988]. It is not unexpected that P2 is active in these cells. The strong evolutionary conservation of P2 can partly account for its predominant activity in many cell lines. P2 is nested within a conserved CpG island [Bangsow et al., 2001; Levanon et al., 2001;

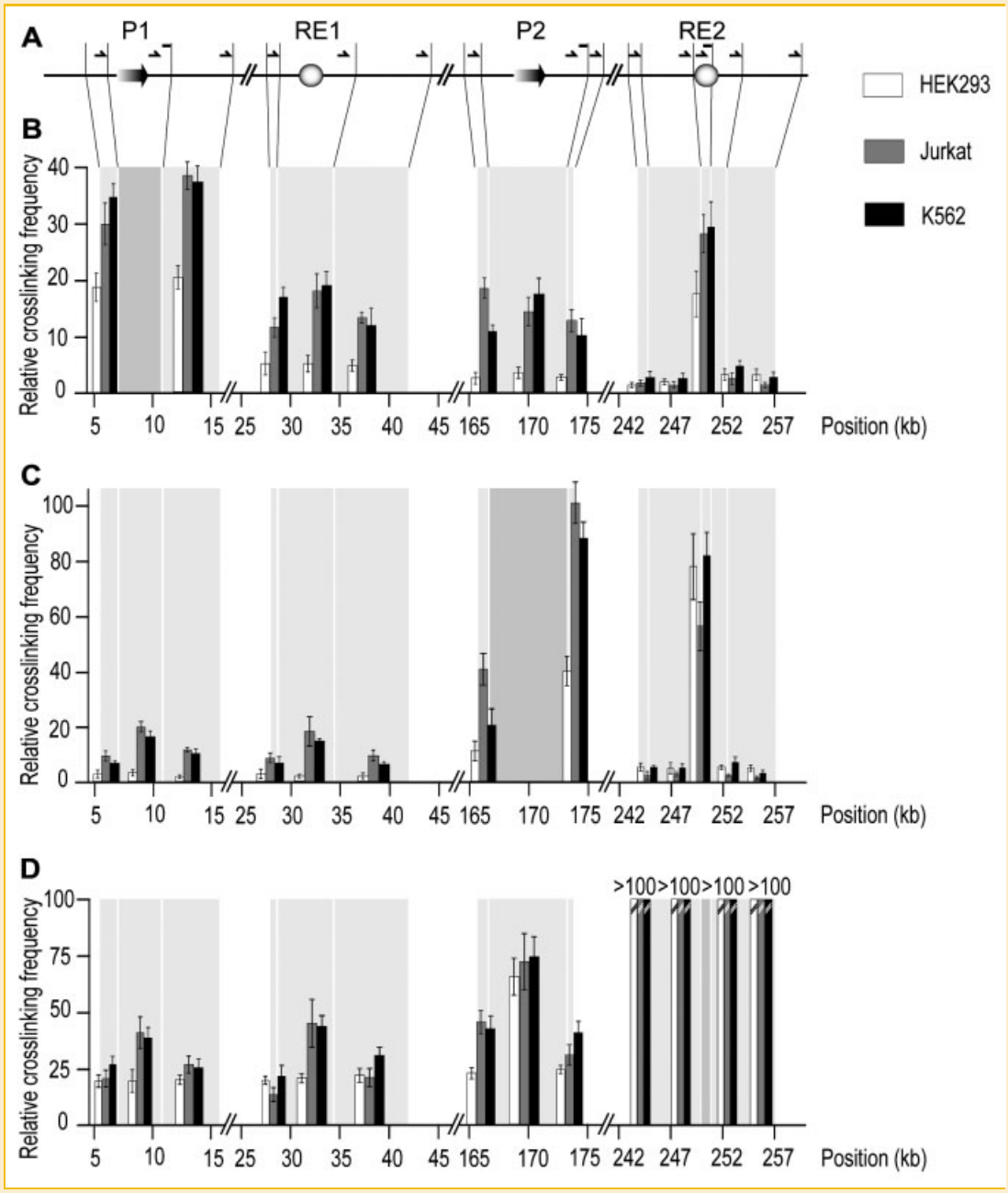

Fig. 4. *Hin*dIII–3C analysis of the spatial organisation of human *RUNX1* gene in lymphoid, erythroid and epithelial cells. A: A scheme depicting the relative positions of P1, P2, RE1 and RE2 (designated by large arrows and beads, not to scale) inside the *Hin*d III restriction fragments, marked by vertical lines. The primers and TaqMan probes used for the 3C analysis are designated by half-arrows and rectangles, respectively. B–D: Relative crosslinking frequencies observed in the experiments with the anchor placed on P1 (B), P2 (C) and RE2 (D). The *x*-axis shows the positions of the restriction fragments on the genomic scale. The light grey rectangles in the background of each diagram indicate the test fragments, and the dark grey rectangles indicate the DNA fragment bearing an anchor. The areas with white background were not analysed. The results obtained for the HEK293, K562 and Jurkat cells are shown by the white, black and grey columns, respectively. The error bars represent SEM for three independent experiments.

Eggers et al., 2002]. However, no such CpG-rich region is found in the vicinity of P1. Furthermore, sea urchins, round worms and fruit flies only have P2, whereas zebrafish, mice and humans have P1 and P2 [Levanon and Groner, 2004]. Vertebrates apparently acquired the extra distal promoter P1, which complicated the mechanism of *RUNX1* gene expression but offered new opportunities for more accurate adjustments of its transcription.

To locate possible regulatory elements, two basic principles are used. The first one, the 'bioinformatical truth' principle, says that regulatory elements are usually evolutionarily conserved and have a high percentage of sequence identity in different species [Flint et al., 2001; Hughes et al., 2005]. The other one, the 'biochemical truth' principle, states that important regulatory elements are located in DHSs [Felsenfeld et al., 1996]. Hence, we chose the two regions of the

RUNX1 gene that satisfied the above-mentioned terms and named them RE1 and RE2. Both of these elements are located in introns and are far from the promoters P1 and P2, especially the RE2 element (Fig. 1). Judging from their position, it was tempting to speculate that RE1 is a specific haematopoietic enhancer that acts on promoter P1 and that RE2 is a specific haematopoietic enhancer that acts on promoter P2. This hypothesis was disproved by the results of the functional test, which showed that RE2 does not have any significant enhancer activity for either of the two *RUNX1* promoters in any of the cell types used. RE1 proved to be a tissue-specific enhancer, acting in lymphoid and erythroid cells, but RE1 functions differently in both cell lines. In lymphoid cells, RE1 was active towards both promoters. In erythroid cells, RE1 was active only towards P2. In HEK293 epithelial cells, RE1 did not show any enhancer activity and was revealed to be a silencer that influenced both P1 and P2 (Fig. 3). Our results show that a tissue-specific enhancer can activate a target promoter in cells of the 'correct' lineage and suppress the same promoter in cells of other lineages. One should not be confused by the relatively low levels of enhancer activities observed in these experiments. The transient transfection experiments cannot fully reproduce the conditions in which promoters and regulatory elements operate when they are placed in the chromosomal context. In the Jurkat cells, the lymphoid cell-specific TCRβ enhancer [Krimpenfort et al., 1988; Levanon et al., 1998; Busse et al., 2005] displayed nearly the same activity as RE1 (Fig. 3).

In general, *cis*-regulatory elements, regardless of their distance from the target promoter, can be brought closer via DNA looping supported by protein–protein interactions. Using the 3C procedure, we demonstrated that both promoters for the *RUNX1* gene are assembled into an active chromatin hub, which also includes RE1 and RE2, in lymphoid (Jurkat) and erythroid (K562) cells. In the HEK293 epithelial cells, we only captured separate interactions between promoters P1 and P2 with RE2 as well as infrequent interactions between RE2 and RE1. In the HEK293 cells, no stable active chromatin hub was likely formed. A dynamic equilibrium exists between the different interactions for all four elements in the cell population with a skewing towards P2-RE2 complex formation. A scheme demonstrating the spatial organisation of the *RUNX1* gene in cells of different lineages is shown in Figure 5. Interestingly, RE2, which possesses no enhancer activity, is present in all types of chromatin hubs identified in our study. This element appears to be essential for the assembly of these hubs, and it can be considered an architectural element that is necessary for the function-dependent three-dimensional organisation of the genome. In lymphoid cells, RE2 likely interacts with some lymphoid-specific regulatory

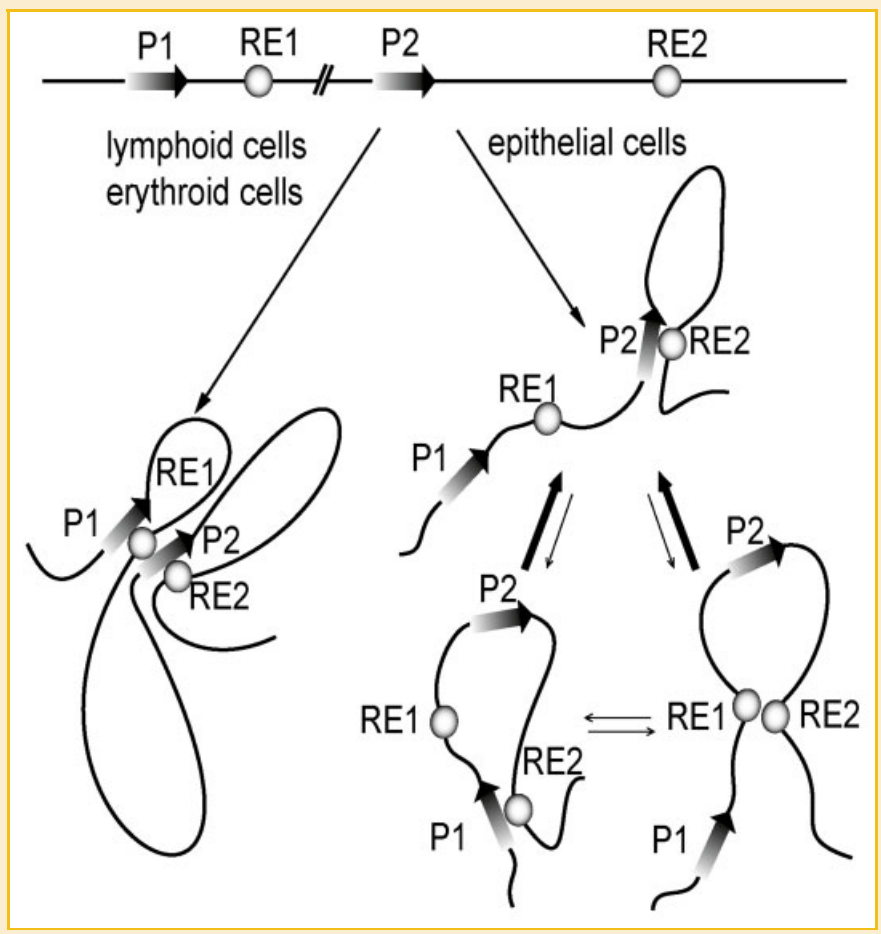

Fig. 5. Model of the human *RUNX1* gene spatial organisation in cells of different lineages (see the text for a description).

proteins based on the presence of lymphoid-specific DHSs within this element. Thus, the fine structure of the *RUNX1* active chromatin hub is not the same in lymphoid and erythroid cells although this hub contains P1, P2, RE1 and RE2 in both cases. An element possessing similar properties, such as RE2, was previously identified in the chicken alpha-globin gene domain. This element (DHS-9) also harbours a tissue-specific DHS and plays a crucial role in the assembly of the active chromatin hub, although it does not possess enhancer activity [Gavrilov and Razin, 2008].

The most interesting feature of the *RUNX1* gene spatial organisation is that the P1 and P2 promoters are recruited to the same chromatin hub in lymphoid and erythroid cells. This recruitment places restrictions on the possibilities of independent regulation of P1 and P2 activity and implies that both promoters are attracted to the same transcription factory. It may happen that in this transcription factory P1 and P2 have to compete for the available RNA polymerase II molecules, and this competition may be an explanation for the different relative levels of P1 and P2 activities observed in erythroid cells in vivo and in transient transfection experiments. Our data (Fig. 2) strongly suggest that most of the mRNA is transcribed from the P2 promoter in the K562 cells. In transient transfection experiments (Fig. 3), both promoters showed comparable basal activity, and the activity of P2 was only moderately increased by RE1. The recruitment of P1 and P2 to the same transcription factory suggests that the nascent transcripts produced, starting from these promoters, are also proximally located. Thus, *trans*-splicing may occur, which could contribute to the complexity of the existing RUNX1 variants.

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