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Control of RUNX2 Isoform Expression: The Role of Promoters and Enhancers

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Abstract The three mammalian *RUNX* genes constitute the family of runt domain transcription factors that are involved in the regulation of a number of developmental processes such as haematopoiesis, osteogenesis and neuronal differentiation. All three genes show a complex temporo-spatial pattern of expression. Since the three proteins are probably mutually interchangeable with regard to function, most of the specificity of each family member seems to be based on a tightly controlled regulation of expression. While *RUNX* gene expression is driven by two promoters for each gene, the promoter sequence alone does not seem to suffice for a proper expressional control. This review focuses on the available evidence for the existence of such control mechanisms and studies aiming at discovering *cis*-acting regulatory sequences of the *RUNX2* gene. J. Cell. Biochem. 95: 506–517, 2005. © 2005 Wiley-Liss, Inc.

Key words: RUNX; transcriptional regulation; transcription factor; *cis*-regulatory genetic element; promoter, enhancer

RUNX2 belongs to the family of runt domain transcription factors. The first runt domain gene to be identified was the pair rule gene *runt* involved in *Drosophila melanogaster* development [Nusslein-Volhard and Wieschaus, 1980]. Runt domain factors have been identified throughout animal phylogenesis with *Caenorhabditis elegans* being the most primitive organism in which a runt orthologue has been detected so far [Nam et al., 2002; Rennert et al., 2003]. Further runt-related orthologues have been identified in invertebrates and vertebrates including the spider *Cupiennius salei*, the beetle *Tribolium castaneum*, the sea urchins *Heliocidaris erythrogramma* and *Strongylocentrotus purpuratus*, fish like *Danio rerio, Oryzias latipes* and *Fugu rubripes*, the frog *Xenopus laevis*, chicken and mammals like mouse, rat and human [Zhu et al., 1994; Castagnola et al., 1996; Coffman et al., 1996; Otto et al., 1997; Haag and Raff, 1998; Tracey et al., 1998; Canon and Banerjee, 2000; Damen et al., 2000; Inohaya and Kudo, 2000; Kataoka et al., 2000; Eggers et al., 2002]. In mammals the runt-related genes are termed *RUNX* [van Wijnen et al., 2004]. Three human and murine *RUNX* genes—*RUNX1* (AML1, CBFA2 and Pebp2αb), *RUNX2* (AML3, CBFA1 and Pebp2αa) and *RUNX3* (AML2, CBFA3 and Pebp2αc)—have been identified and localised on human chromosomes 21q22.12, 6p21 and 1p36.1 and mouse chromosomes 16, 17 and 4 respectively [Bae et al., 1994; Levanon et al., 1994; Avraham et al., 1995; Calabi et al., 1995; Otto et al., 1997; Zhang et al., 1997].

RUNX2 is a transcription factor essential for skeletal development. It is indispensable for osteoblast differentiation and hence for bone formation. This has been shown by a large number of in vitro experiments and impressively confirmed by the complete absence of ossification in Runx2 knockout mice [Geoffroy et al., 1995; Ducy et al., 1997; Komori et al., 1997; Otto et al., 1997]. The importance of RUNX2 for bone formation is furthermore demonstrated by inactivating mutations of the *RUNX2* gene that lead to the autosomal dominant skeletal disorder cleidocranial dysplasia (CCD) [Mundlos et al., 1997; Quack et al., 1999]. Interestingly, heterozygous Runx2 knockout mice exhibit a skeletal phenotype that strongly resembles that of human CCD and *Ccd* mice—a mouse model for CCD—harbour a large deletion

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on chromosome 17 including the *Runx*2 gene [Selby and Selby, 1978; Otto et al., 1997]. The physiological properties of RUNX2, however, are not limited to bone development. RUNX2 has furthermore been characterised as an important factor in chondrocyte maturation. Runx2 is expressed in cartilage and Runx2 null mice exhibit perturbed chondrocyte maturation [Otto et al., 1997; Kim et al., 1999]. Involvement of RUNX2 in chondrocyte differentiation was also demonstrated in cell culture experiments and in transgenic mice expressing a dominant negative Runx2 mutant selectively in cartilage [Enomoto et al., 2000; Stricker et al., 2002]. In addition, expression of RUNX2 was detected in thymus and testis [Satake et al., 1995; Ogawa et al., 2000]. Until now the functional relevance of RUNX2 expression in the latter tissues has not yet been clarified.

Expression of RUNX2 is initiated from two promoters, the distal P1 promoter and the proximal P2 promoter, separated by exon1 and a large intron. These promoters give rise to two major protein isoforms with distinct aminotermini [Xiao et al., 1998]. The physiological implications and relevance of these two isoforms have been lively discussed in recent years, warranting a concise summary of our current knowledge about RUNX2 isoform expression [Xiao et al., 1999, 2004; Banerjee et al., 2001].

In this review we will focus on the control of RUNX2 isoform expression and the *cis*-regulatory elements involved in this regulation.

GENOMIC ORGANISATION OF THE *RUNX2* GENE

The genomic and cDNA structure of the three mammalian paralogous *RUNX* genes is highly conserved [Levanon et al., 1994, 2003b; Bangsow et al., 2001; Coffman, 2003; Rennert et al., 2003]. The similarity between the mammalian *RUNX* paralogues extends even beyond the coding sequence. All three genes have two promoters, the distal P1 promoter and the proximal P2 promoter [Ogawa et al., 1993; Ghozi et al., 1996; Stewart et al., 1997; Xiao et al., 1998; Fujiwara et al., 1999; Rini and Calabi, 2001]. Moreover homology includes the neighbouring paralogous genes CLIC and DSCR [Levanon et al., 2001b, 2003a; Eggers et al., 2002]. The similarity in protein structure is reflected by the ability of all three RUNX factors to regulate the bone sialoprotein (BSP) promoter [Ducy et al., 1997; Javed et al., 2001; Otto et al., 2003].

The human *RUNX*2 gene spans approximately 220 kb [Levanon et al., 1994]. It consists of at least eight exons that have been numbered in different ways by different authors [Geoffroy et al., 1998; Xiao et al., 1998; Quack et al., 1999]. In order to establish an exon nomenclature that provides consistent numbering among the paralogous *RUNX* exons, we previously proposed the numbering indicated in Figure 1 [Otto et al., 2002].

Two distinct promoters drive expression of two major RUNX2 isoforms. At the distal promoter P1 type II isoform expression is initiated, while type I isoform transcription is initiated at the proximal promoter P2 type [Xiao et al., 2001]. Type I (P2) Runx2 was originally cloned as a T-cell specific factor: Pebp2αA [Ogawa et al., 1993; Satake et al., 1995]. However, this isoform is also expressed in other non-osseous tissues and in osteoblasts [Harada et al., 1999]. Type II (P1) Runx2 was originally cloned as a bone-specific factor: Osf-2; til-1 [Ducy et al., 1997; Mundlos et al., 1997; Stewart et al., 1997].

A third potential N-terminal isoform has been described by Ducy et al. [1997] in the mouse. In this case an alternative translation start in exon 1 results in a type II isoform protein with additional 87 N-terminal aminoacids starting with the sequence MLHSPH. Functional relevance of this alternative translation start, however, has not been demonstrated and in transfection experiments cDNAs with or without the sequences responsible for this MLHSPH did not reveal gross functional differences [Xiao et al., 1999]. Moreover, this sequence is not conserved in the human *RUNX*2 gene [Lee et al., 1997; Xiao et al., 1998]. Further isoforms result from alternative splicing [Geoffroy et al., 1998; Xiao et al., 1998; Ogawa et al., 2000]. This seems to be a common feature of the runt-related genes, since multiple isoforms have also been detected for RUNX1 [Bae et al., 1994; Levanon et al., 1996; Tsuji and Noda, 2000].

The proteins deriving from the two major isoforms share the same functional domains like glutamine–arginine-rich domain (QA), DNA-binding runt domain (RHD), nuclear localisation signal (NLS), nuclear matrix targeting signal (NMTS) and the TLE/groucho interacting carboxyterminal pentapeptide VWRPY [Lindenmuth et al., 1997; Imai et al., 1998; Thirunavukkarasu et al., 1998; Quack et al.,

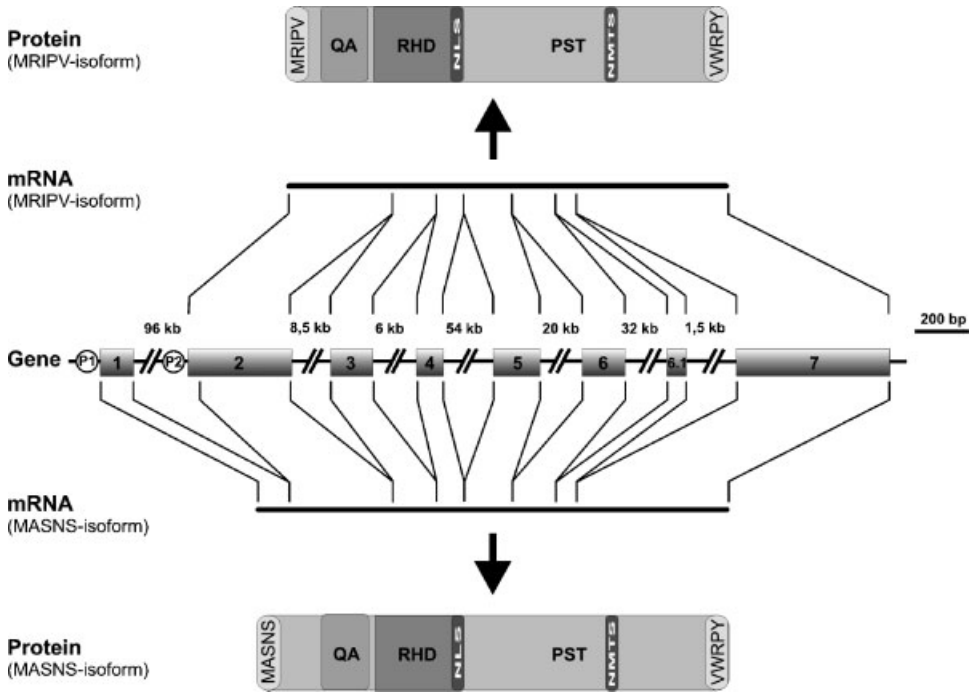

Fig. 1. Genomic organisation and functional protein structure of human RUNX2. The 220 kb stretching human *RUNX2* gene is located on chromosome 6p21. It contains eight exons termed exon 1, 2, 3, 4, 5, 6, 6.1 and 7. Intron sizes are indicated. Initiation of transcription at the distal P1 promoter results in the expression of the bone specific isoform with the N-terminal pentapeptide MASNS. Transcription of the more widely expressed second major isoform with the N-terminal pentapeptide MRIPV is produced by usage of the proximal P2 promoter. QA, QA domain; RHD, runt homology domain; NLS, nuclear localisation signal; PST, proline/serine/threonine rich region; NMTS, nuclear matrix targeting signal; VWRPY, TLE interacting domain.

1999; Javed et al., 2000; Zaidi et al., 2001]. However, one of three activation domains defined in vitro using reporter assays has been mapped to the aminoterminus of type II RUNX2 and is not present in RUNX2 type I [Thirunavukkarasu et al., 1998]. Whether this difference leads to a different transactivation potential of the two major RUNX2 protein isoforms has been controversially discussed [Xiao et al., 1999; Banerjee et al., 2001]. The genomic and protein isoform structures are illustrated in Figure 1.

EXPRESSION OF RUNX2 ISOFORMS

Despite the high structural similarity, the mammalian RUNX paralogues fulfil distinct physiological tasks. RUNX1 plays an essential role in hematopoesis, RUNX2 is needed mainly for skeletal development and RUNX3 is involved in neurogenesis, thymopoesis and growth behaviour of gastric epithelium [Okuda et al., 1996; Wang et al., 1996; Komori et al., 1997; Otto et al., 1997; Inoue et al., 2002; Levanon et al., 2002; Taniuchi et al., 2002; Woolf et al., 2003]. The difference in physiologic properties of the three RUNX factors is thought to be caused at least to a large degree by the distinctive expression patterns of these transcription factors [Levanon et al., 1994, 2001a; Otto et al., 1997]. However, in fetal thymus all mammalian RUNX paralogues are expressed [Satake et al., 1995; Levanon et al., 1996, 2001a; Komori et al., 1997; Hayashi et al., 2000]. Yet, a potential cellular co-expression in thymus only occurs early in development. At later stages the different paralogues are restricted to different compartments of the thymus [Levanon et al., 2001a; Taniuchi et al., 2002; Woolf et al., 2003]. Overlapping expression patterns of the RUNX paralogues have also been observed in the skeleton [Simeone et al., 1995; Kim et al., 1999; Enomoto et al., 2000; Levanon et al., 2001a; Stricker et al., 2002; Yamashiro et al., 2002; Lian et al., 2003].

The distinct expression pattern of the RUNX paralogues is thought to be mainly controlled by the two promoters. RUNX2 isoform expression

has been investigated by Ducy et al., Geoffroy et al. [1998] and Xiao et al. [1998] amongst others. Initial experiments showed that the P1-derived type II isoform is bone-specific. First evidence for this hypothesis came from RT-PCR data [Ducy et al., 1997]. Enomoto et al. [2000], however, found that both P1 and P2 isoforms are expressed in bone and terminal hypertrophic cartilage. Additionally, they found P2-derived Runx2 also expressed in earlier hypertrophic and pre-hypertrophic chondrocytes. These results were obtained by in situ hybridisation. Other studies showing mRNA expression of both isoforms in osteoblastic cells and osteoblast precursors supported these findings. In the latter studies the predominantly expressed isoform in non-osteoblastic cells was RUNX2 type I [Sudhakar et al., 2001b]. Yet, other investigators could not detect Runx2 type I in osteoblasts [Xiao et al., 1998]. Another study supporting type II being the osteoblast-type Runx2 investigated the isoform expression in cranial suture morphogenesis. While type I isoform was most intensely expressed in the sutural mesenchyme, type II isoform was predominantly expressed in the osteogenic fronts of the calvariae [Park et al., 2001]. Furthermore, the P1-derived type II isoform has been shown to be highly expressed in differentiating osteoblasts and responsive to bone morphogenetic protein (BMP) 2. Type II isoform was upregulated during osteogenic differentiation and its expression was absent from non-osseous tissues. The P2-derived type I isoform in contrast was constantly expressed in osteoblastic, pre-osteoblastic and even in non-osteoblastic mesenchymal cells. Expression of P2 Runx2 was not altered during osteogenesis and only P1 Runx2 expression was elevated following administration of BMP-2. The two isoforms exhibited similar though not identical biological activity on the promoters of several target genes [Banerjee et al., 2001]. Xiao et al. [1999], however, reported a higher transactivation potential of Runx2 type II as compared to type I under certain conditions.

Analysis of isoform expression at the protein level revealed that type I Runx2 protein was expressed in early and mature osteoblasts while Runx2 type II protein was only expressed in mature osteoblasts. These observations were assessed by cell culture experiments [Sudhakar et al., 2001a]. Post-transcriptional events have been reported to contribute to the regulation of *Runx*2 gene expression. To this end, Sudhakar et al. have shown that while mRNA for both Runx2 isoforms is expressed in osteoblastic and pre-osteoblastic cells as well as in non-osteoblastic cells Runx2 protein expression is more specific. In non-osteoblastic cells and osteoblastic precursors they could not detect any Runx2 protein isoform. Moreover, while they could detect isoform I Runx2 protein only in less mature osteoblasts, in mature osteoblasts both protein isoforms were expressed. Runx2 protein expression correlated with a physical association of the respective mRNA with polysomes. The authors suggest that dormant mRNAs in osteoblastic precursors become activated during differentiation [Sudhakar et al., 2001b]. Support for this hypothesis comes from a study showing an increase in RUNX2 protein expression in human pre-osteoblasts after administration of dexamethasone without an increase in RUNX2 mRNA levels [Prince et al., 2001]. Translational control has also been reported to be a crucial checkpoint in the expression of RUNX1 protein [Pozner et al., 2000].

To summarise these studies it is generally accepted that RUNX2 type II represents the highly regulated isoform intensely expressed in mature osteoblasts and terminally differentiated hypertrophic chondrocytes, while type I RUNX2 is thought to be a more broadly expressed isoform, present also in earlier precursors of osteoblasts and chondrocytes.

However, this model might be only of limited validity. In order to investigate the importance of RUNX2 type II for bone development Xiao et al. generated a mouse deficient in type II Runx2 by targeted disruption of the P1 promoter. Surprisingly, bone formation is only weekly affected in Runx2 type II null mice. Disturbance of bone formation in these mice is predominantly confined to endochondral ossification [Xiao et al., 2004]. In contrast, heterozygous total Runx2 KO mice exhibit impairments predominantly in intramembranous bone formation [Otto et al., 1997]. This implies that in Runx2 type II null mice the skeletal phenotype might not be caused simply by Runx2 insufficiency. It also shows that bone formation is not exclusively mediated by RUNX2 isoform type II, and that type I RUNX2 can substitute at least to some extend for RUNX2 type II. However, calvarial osteoblasts derived from Runx2 Type II deficient mice exhibited impaired expression of osteoblast markers, and these expression

levels could be rescued by ectopic introduction of both Runx2 I or Runx2 II in reporter assays [Xiao et al., 2004].

Mice made deficient in the expression of the homeobox transcription factor Bapx1 show perturbations in skeletal formation and spleen development. The skeletal malformations are most severe in the axial skeleton, which is formed by endochondral ossification. Moreover, Bapx1 null mice exhibit significantly lower levels of Runx2 expression [Tribioli and Lufkin, 1999]. In this respect the phenotypes of Bapx1 null and Runx2 type II-deficient mice overlap to a degree. This leads to the assumption that Bapx1 dependent regulation of *Runx*2 gene expression affects predominantly type II Runx2. Interestingly, potential *cis*-regulatory elements outside of the promoter regions conserved between pufferfish and human contain consensus binding sites for homeobox factors [Eggers et al., 2002]. Thus, it is tempting to speculate that the homeobox transcription factor Bapx1 might activate Runx2 expression by binding to these conserved genetic elements, that may serve as enhancers.

In contrast to total Runx2 KO mice chondrocyte development and maturation was not grossly altered in Runx2 type II deficient mice. The hypertrophic cartilage zone was widened possibly as a result of delayed and impaired endochondral ossification or due to decreased levels of matrix remodelling enzymes like MMP9 and MMP13. Furthermore increased levels of Sox9, Coll II and Coll X indicate an enhanced chondrogenesis [Xiao et al., 2004]. Interestingly Sox9 was decreased in Runx2 overexpressing C3H10T1/2 embryonic fibroblasts [Stock et al., 2004]. These findings point to a regulatory interdependence between developing osteoblasts and chondrocytes.

Taken together, the question whether the two Runx2 isoforms have distinct biological functions in vivo has gained even more relevance recently.

REGULATION OF RUNX2 EXPRESSION BY THE TWO *RUNX*2 PROMOTERS

The *RUNX*2 promoters are highly conserved between different mammalian species (mouse, rat, human) and even the puffer fish (*Fugu rubripes*) *frunx2* promoters are very similar [Drissi et al., 2000; Eggers et al., 2002]. This is functionally reflected by the finding that the P1 promoter of all three mammalian RUNX paralogues could be activated by the integration of the murine leukemia virus subsequently leading to the development of T-cell lymphoma in CD2-Myc transgenic mice [Stewart et al., 1997, 2002; Cameron et al., 2003].

The transcription start of type II Runx2 is located approximately 400 bp upstream the translation start, thus defining the border between promoter and 5′-untranslated region (5′-UTR) [Geoffroy et al., 1998; Drissi et al., 2000]. A 1.4 kb fragment of the P1 promoter exhibited promoter activity in several cell lines. Only marginal differences in activity were obtained in mesenchymal lines like NIH3T3, L929 fibroblasts, C3H10T1/2 and strongly Runx2 expressing MC3T3-E1 cells. In Cos-7 cells, however, the promoter was silent. This study furthermore examined the transcription start and exon1 of Runx2 type II. A mini-intron was discovered that splits exon1. A second minor transcription start site 418 nucleotides upstream of the previously defined major transcription start was also identified [Geoffroy et al., 1998; Drissi et al., 2000; Xiao et al., 2001]. Other studies of the P1 promoter revealed that a fragment containing a mere 0.6 kb of sequence upstream of the transcription start is sufficient for promoter activity. The P1 promoter was organised in two regulatory regions highly conserved between mouse, rat and human. In rat the distal regulatory region ranged from −458 to −304 (containing a repressive element at −458 to −451) and the proximal domain from −113 to −1. Like in the human and mouse promoters these domains were separated by a purine rich region. While the main activating sequences were localised between −351 and −92 (in rat), the regions downstream of −92 and the 5′-UTR contain repressive elements. The authors found evidence for a negative autoregulation of *Runx*2 P1. Interestingly, a major decrease in promoter activity was observed by deleting the purine-rich region in serial deletion analyses, a more severe effect than deleting the two defined regulatory regions. Six binding sites for RUNX have been detected within promoter P1 and 5′-UTR. Reporter assays revealed a repressing effect of ectopic Runx2 expression on the P1 promoter [Drissi et al., 2000]. However, the constructs the investigators used for reporter assays were based on pGL3, a luciferase reporter vector that has been reported to respond in reporter activity to Runx2 expression due to

RUNX binding sites intrinsic to the vector [Thirunavukkarasu et al., 2000] (and Stock et al., unpublished data). Furthermore, the studies by Ducy et al. yielded conflicting results. They reported the suppression of murine Runx2 expression and P1 promoter activity by a dominant negative Runx2 mutant [Ducy et al., 1999].

All these studies investigated the promoter activity of P1 in vitro. In summary they demonstrated that a fragment as short as 0.6 kb and flanking the gene towards the 5′ end was enough to confer substantial promoter activity in osteoblastic cells like ROS17/2.8 or MC3T3-E1 [Drissi et al., 2000]. In vivo however, a 3 kb fragment containing the P1 promoter cloned in 5′ orientation with regard to the β-Gal gene exhibited promoter activity in transgenic mice only in mesenchyme, committed chondroprogenitors, pre-chondrocytes and mature chondrocytes, depending on developmental stage of the embryo. Hypertrophic chondrocytes and osteoblasts did not reveal any P1 promoter activity [Lengner et al., 2002]. These observations unveil the limitations of in vitro experiments in the study of *cis*-regulatory elements.

For protein-promoter interactions, however, we can only rely on in vitro data, so far. Both *RUNX*2 promoters harbour several RUNX consensus binding sites. This attribute is also conserved in the promoters of the other RUNX paralogues [Ghozi et al., 1996; Ducy et al., 1999; Drissi et al., 2000, 2002b; Bangsow et al., 2001]. These findings and the fact that all RUNX paralogues bind to the same consensus DNA sequence imply the possibility of both RUNX auto-regulation and cross-regulation of the different RUNX paralogues [Drissi et al., 2000; Otto et al., 2003; Levanon and Groner, 2004]. Different in vitro studies have assigned either positive or negative regulatory potential to the highly conserved RUNX binding sites within the *RUNX*2 P1 promoter [Ducy et al., 1999; Drissi et al., 2000; Alliston et al., 2001]. The in vivo model of Runx2 type II null mice, however, shows an incomplete compensation of total Runx2 levels by increased expression of Runx2 type I [Xiao et al., 2004]. Thus, this model supports negative auto-regulation of the *Runx2* gene as proposed earlier, however mediated by the P2 promoter [Drissi et al., 2000].

The perfectly conserved region at −92 to −78 bp of the P1 *RUNX*2 promoter has been shown to include a vitamin D response element (VDRE) that interacts with VDR/RXR heterodimers, thus directly mediating suppressive effects of 1,25 dihydroxyvitamin D3 on RUNX2 expression [Drissi et al., 2002a]. The same region harbours binding sites for RUNX and AP1. In vitro the latter mediate response to selective estrogen receptor modulators (SERMs). In vivo, however estrogen responsiveness could not be demonstrated [Tou et al., 2001].

Zambotti et al. [2002] identified a functional enhancer element within the mouse P1 promoter. This element, termed CE1, is located in a region between −415 and −375. It contains binding sites for AP1 and NF1 factors. NF1-A, usually not present in osseous tissues, appeared to bind to the element and repress transcription. CE1 could be cloned adjacent to a different promoter and still acted as an enhancer [Zambotti et al., 2002].

Members of the TGFβ superfamily have been shown to regulate RUNX2 expression. TGF-β has been reported to enhance RUNX2 expression in the pre-myoblastic multipotent cell line C2C12, while repressing its expression in primary calvarial cells or the rat osteosarcoma cell line ROS17/2.8 [Li et al., 1998; Lee et al., 1999, 2000; Alliston et al., 2001]. These findings correlate with data showing that TGF-β enhances early osteoblast development while it inhibits terminal osteoblast differentiation [Bonewald and Dallas, 1994; Centrella et al., 1994]. SMADs, the intracellular transducers of the TGF-β signal have been shown to physically interact with RUNX2 [Zhang et al., 2000; Ito and Zhang, 2001]. Thus, TGF-β responsive elements might be represented by the RUNX binding sites present in both P1 and P2 promoters. BMP4/7 heterodimers have been demonstrated to induce Runx2 expression in immature mesenchymal C2C12 or MC3T3-E1 cells and BMP2 was shown to stimulate specifically the expression of RUNX2 type II in C3H10T1/2 [Tsuji et al., 1998; Banerjee et al., 2001]. However, a direct influence of BMP2 on the activity of a 1.4 kb P1 promoter fragment could be detected. So far the BMP2 responsive element has not been identified [Xiao et al., 2001].

Further external factors found to participate in *RUNX*2 gene regulation but mediating this control via so far unknown response elements include members of the hedgehog family of signalling molecules. Administration of sonic hedgehog (Shh) to murine embryonic fibroblasts C3H10T1/2 resulted in stimulation of

Runx2 mRNA expression. This correlated with an activation of the P1 promoter, however a Shh responsive element has not been identified [Spinella-Jaegle et al., 2001; Takamoto et al., 2003].

While these studies point to a highly regulated P1 promoter and a rather constitutively active P2 promoter with a constant basal transcription rate, data revealing a regulatory effect of TNFα on Runx2 expression show that also the P2 promoter has regulatory potential. In these studies the investigators show that TNFα represses Runx2 expression predominantly by inhibiting transcription from the P2 promoter [Gilbert et al., 2002]. The P2 promoter is furthermore located within a large CpG island that extends to the 5′-UTR of exon 1. This feature is conserved among the mammalian *RUNX* paralogues and even in the *RUNX2* orthologue of pufferfish (*Fugu rubripes*) *frunx2* [Bangsow et al., 2001; Levanon et al., 2001b; Eggers et al., 2002; Levanon and Groner, 2004]. For *RUNX2* the physiologic role of this CpG island has not been determined, yet. However, for the *RUNX3* locus this CpG island has been discussed to be hypermethylated with concomitant silencing of RUNX3 expression in several types of malignancies including gastric, lung and colorectal cancer [Li et al., 2002, 2004; Ku et al., 2004]. Another highly conserved CpG island is located at the 3′ end of the gene [Bangsow et al., 2001; Levanon et al., 2001b; Levanon and Groner, 2004]. Figure 2 summarises the organisation of the well-characterised P1 *RUNX2* promoter.

The studies on the *RUNX2* promoters summarised here can only partially explain the highly specific time- and tissue-dependent expression pattern of this gene. This is particularly demonstrated by discrepancies in data from in vivo studies compared to those obtained from in vitro analyses. Thus, it seems likely that further *cis*-regulatory elements contribute to *RUNX2* gene regulation in vivo.

SEARCHING FOR ENHANCERS CONTRIBUTING TO THE REGULATION OF *RUNX2* GENE EXPRESSION

Our group searched the *Runx2* flanking regions for potential *cis*-regulatory sequences. The highly conserved homology among the runt-related genes of different species implies the possibility that regulatory DNA stretches might also be conserved [Rennert et al., 2003]. Therefore, the *RUNX2* orthologue of the pufferfish *Fugu rubripes*, *frunx2*, was cloned and sequenced. A comparison of the genomic loci of *RUNX2* and *frunx2* revealed three highly conserved regions in addition to the coding and promoter regions of the genes. These elements, containing consensus binding sites for RUNX, HOX, SMAD, SOX and AP-1 factors, may have a *cis*-regulatory role. These conserved elements are located within the intronic sequence 5′ to exon 6.1, and approximately 200 and 400 kb (in human) upstream of exon 1 respectively [Eggers et al., 2002] (Fig. 3). Although at least the latter two elements are located at quite some distance from the *RUNX2* promoter they may represent missing pieces in the puzzle of *cis*-regulatory elements that are essential for the expression of Runx2 type II in osteoblasts and terminally differentiated chondrocytes. A distance of 400 kb from the transcription start is not uncommon for long-range enhancers. For example, an enhancer for Shh has been identified approximately 1 Mb upstream of the *Shh* gene

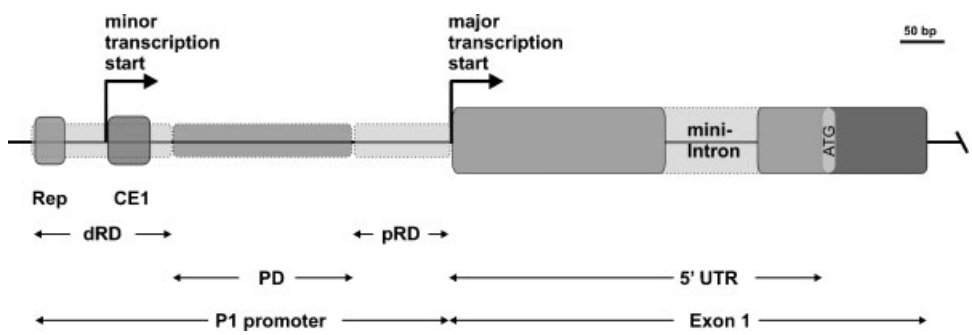

Fig. 2. Organisation of the distal *RUNX2* promoter (P1). Major and minor transcription start and translation start (ATG) are indicated. 5′ UTR, 5′ untranslated region; CE1, enhancer element; dRD, distal regulatory domain; pRD, proximal regulatory domain; PD, purine-rich domain; Rep, repressive element.

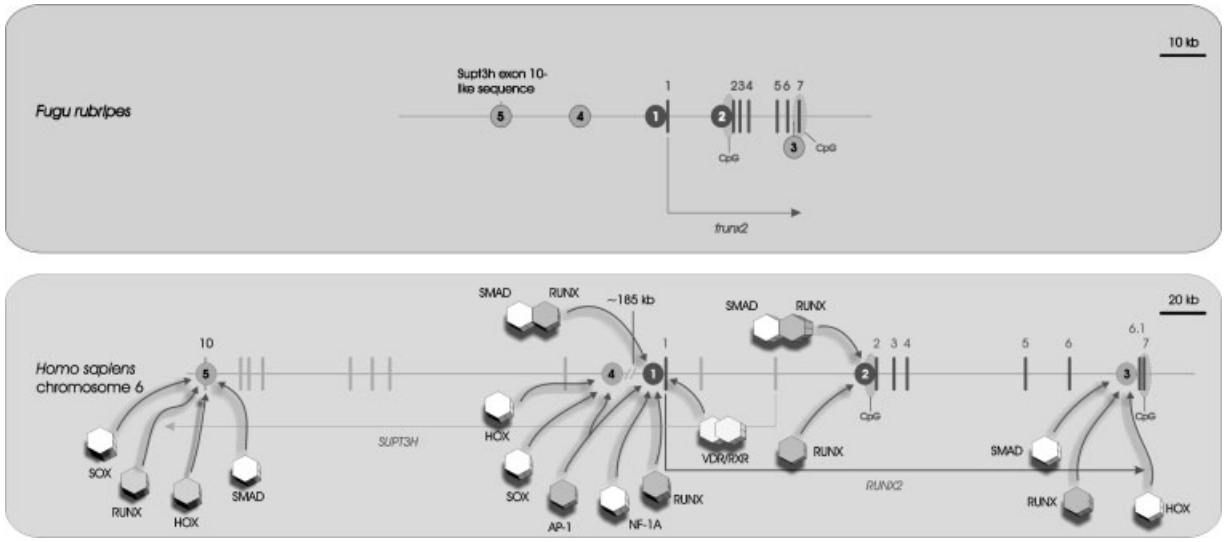

Fig. 3. Schematic comparison of the *RUNX2* orthologues of *Fugu rubripes* and *Homo sapiens*. The genomic organisation of Fugu and human *RUNX2* orthologues and their flanking regions are displayed. Exons are indicated as numbered vertical lines. Conserved non-coding regions are shown as numbered circles: 1 and 2 represent RUNX2 promoters 1 and 2 respectively. 3, 4 and 5 correspond to conserved regions potentially serving as additional *cis*-acting genetic elements. Potential binding sites within conserved regions 1–5 for different transcription factors are also indicated. Vertical lines in light grey indicate exons of the 5′ neighbouring gene suppressor of Ty 3 homolog (*SUPT3H*). CpG: CpG island.

and it is located within another gene [Lettice et al., 2002]. Nobrega et al. [2003] identified several long-range enhancers for the human DACH gene. The most distant enhancer element was 780 kb upstream of the gene

Concerning the existence of long-range enhancers and the fact that the *Runx2* promoter P1 alone is not capable of driving gene expression in hypertrophic chondrocytes and osteoblasts it is warranted to search for novel *cis*-acting elements contributing to the regulation of *RUNX2* gene regulation [Lengner et al., 2002; Lettice et al., 2002; Nobrega et al., 2003]. The decryption of whole genomes including that of the mouse has provided the chance to apply comparative genomics to the search for *cis*-regulatory elements. Thus, comparative analysis of the *Sox9* loci of pufferfish and human identified several enhancer elements up to 290 kb upstream of the gene [Bagheri-Fam et al., 2001]. In the case of the human *DACH* gene long-range enhancers have been identified by comparing the *DACH* loci and neighbouring sequences of human, mouse, frog and pufferfish [Nobrega et al., 2003]. These examples show that comparative genomics is a feasible method to identify long-range *cis*-regulatory elements that are essential for fully functional control of *RUNX2* expression. The recently released chicken genome might simplify the search for these elements, since it adds another model organism to compare genomic loci with.

We have performed a preliminary in vitro analysis of elements of the *Runx2* gene that have been identified using a comparative genomics approach. Initial data of these studies indicate that at least some of these elements can act as enhancers. Currently, these sequences are being tested in vivo and results are expected in the near future. An identification of functional enhancer elements will hopefully provide novel insight into how different functions of the *Runx* genes exerted despite the fact that the proteins are structurally so similar.

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