

The artificial zinc finger protein 'Blues' binds the enhancer of the fibroblast growth factor 4 and represses transcription

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Abstract The design of novel genes encoding artificial transcription factors represents a powerful tool in biotechnology and medicine. We have engineered a new zinc finger-based transcription factor, named Blues, able to bind and possibly to modify the expression of fibroblast growth factor 4 (FGF-4, K-fgf), originally identified as an oncogene. Blues encodes a three zinc finger peptide and was constructed to target the 9 bp DNA sequence: 5'-GTT-TGG-ATG-3', internal to the murine FGF-4 enhancer, in proximity of Sox-2 and Oct-3 DNA binding sites. Our final aim is to generate a model system based on artificial zinc finger genes to study the biological role of FGF-4 during development and tumorigenesis.

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1. Introduction

Various strategies have been reported for generating new artificial transcription factors able to recognize and to modify the expression of a specific gene of interest [1,2]. The zinc fingers of the class Cys2-His2 appear optimal for this purpose, thanks to their plasticity and versatility [1–5]. The X-ray crystal structures of the three zinc finger transcription factor Zif268 bound to its DNA target reveal that each individual finger binds essentially 3 bp of double-stranded DNA, through specific contacts with the amino-terminal part of the α -helix (amino acid positions: –1, +3, +6) [6,7]. In particular, a 'recognition code' that relates the amino acids of a single finger to its associated subsite DNA target has been proposed for a variety of finger domains [8–14]. Rational zinc finger design, using the code and/or the selection approach, permits the production of finger domains with satisfactory binding properties to target almost any desired sequence in the genome [15]. The fusion of the artificial zinc finger DNA binding domain with different transcriptional regulatory domains can result in either activation or repression of the expression of chosen target genes [3,14,16,17]. Several research groups have produced synthetic transcription factors

able to repress the expression of specific oncogenes. Choo and Klug have constructed a three finger peptide that binds the unique sequence, 9 bp long, present in the junction of the c-Abl proto-oncogene and BCR gene, in acute lymphoblastic leukemia [18]. Barbas and colleagues have recently realized finger chimeric proteins able to modulate the expression of the genomic loci of erb-2 and erb-3 genes, both involved in human cancers [19,20].

The fibroblast growth factor 4 (FGF-4) was originally identified as an oncogene, by transfection experiments of DNA derived from Kaposi's sarcoma [21,22]. The mechanism of activation of the proto-oncogene is due to unregulated expression, rather than the production of an abnormal protein [23]. Generally, a gene encoding a growth factor can potentially behave as an oncogene in any cell type that does not strictly regulate its expression. The physiological expression of the murine proto-oncogene FGF-4, silent in the adult, is restricted to early embryonic stages, specifically in the inner cell mass of blastocyst and later in specific embryonic tissues [24–26]. The growth factor FGF-4 has been shown to be essential for post-implantation mouse development by knock-out experiments [27]. Assuming that FGF-4 expression patterns in human and mouse are similar, no expression would be expected in normal human adult tissues. Nonetheless, expression of FGF-4 and INT2 (FGF-3) has been detected in Kaposi's sarcoma, whose cytogenetic and molecular bases are still poorly understood. Genomic co-amplification of these two genes has also been reported in different human malignancies, including breast cancer and melanoma [28].

Accordingly to its physiological pattern of expression, FGF-4 is observed in murine embryonic stem cells and embryonic carcinoma (EC) cell lines. FGF-4 is absent in HeLa and NIH3T3 cell lines [24]. In a mouse F9 teratocarcinoma cell line, FGF-4 is switched off upon differentiation. In EC cells FGF-4 expression requires the synergistic interaction between the two transcription factors Oct-3 and Sox-2, on the FGF-4 enhancer [29–32]. In particular, Sox-2 and Oct-3 bind to adjacent *cis*-acting elements to form a ternary protein–DNA complex [30]. The FGF-4 enhancer, located in the 3' untranslated region, is conserved both in human and murine genes and is able to drive transcription starting from both homologous and heterologous promoters [24].

Here, we demonstrate that the engineered gene Blues encodes a zinc finger protein (pBlues) that specifically binds its chromosomal DNA target. The Blues gene product is able to modulate transcription of a test gene, from the FGF-4 enhancer.

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2. Materials and methods

2.1. Constructs

The construction of the Blues gene was obtained as described by Corbi et al. [12–14]. Briefly, using as a model the zinc finger backbone of the Zif268 gene [33], we synthesized two overlapping oligonucleotides, named Blues-5' (154 nt) and Blues-3' (160 nt) respectively. The resulting Blues DNA fragment was cloned both into the pRK5-HA mammalian expression vector (pRK5-Blues), containing the hemagglutinin (HA) tag sequences, and into the pGEX-4T-3 bacterial expression vector (GST-Blues) (Pharmacia). To construct the pRK5-Krab-Blues expression vector the Blues zinc finger domain was cloned at the 5' end of the Kruppel-associated box A (KRAB A) repression domain derived from the murine Zfp60 gene (amino acids 1–54, GenBank accession number U48721) (Fig. 4A) [34]. The reporter plasmid pGL3PrBTS(5n) was constructed cloning five copies of monomer BTS sequence at the 5' end of the SV40 promoter into pGL3 Promoter vector (Promega).

2.2. Expression of bacterial recombinant fusion protein

The recombinant GST-Blues protein was expressed in BL21(DE3) host bacteria by isopropyl- β -D-thiogalactose induction and purified using glutathione-agarose beads as previously described [14].

2.3. Electrophoretic mobility shift assay (EMSA)

EMSA was performed as previously described [14]. The oligonucleotide probes contained either one or two copies of BTS target (underlined): 5'-TTTCGCTCGGGCTCGAGGGTTTGGATGGGC-TCGAGGTCAGG-3' and 5'-TCGACGGTTTGGATGGGCTCGAGGGTTTGGATGGGCTCGAGG. The DNA sequences of the mutagenized oligonucleotides BTSm1, BTSm2 and BTSm3 were: 5'-TTTCGCTCGGGCTCGAGGACCTGGATGGGCTCGAGGTCAGG-3', 5'-TTTCGCTCGGGCTCGAGGGTTATAATGGGCTCGAGGTCAGG-3' and 5'-TTTCGCTCGGGCTCGAGGGTTTGGTTTGGCTCGAGGTCAGG-3' respectively. The oligos used in EMSA were labeled using T4 polynucleotide kinase. Supershift of Blues protein was obtained adding 3 μ l of mouse monoclonal antibody (clone 12CA5) to the HA peptide derived from the influenza hemagglutinin protein (Roche Diagnostic).

To measure the apparent dissociation constant [12–14] (K_d) of the GST-Blues/BTS complex we performed EMSA [14]. The radioactive signals were visualized by autoradiography and quantitated by ImageQuant software (Molecular Dynamics) and the data were analyzed with the KaleidaGraph program (Abelbeck Software). The K_d value was determined as an average of three separate studies. Small-scale nuclear extracts from mammalian cell lines were prepared as previously described [14]. EMSAs were performed using 2–5 μ g of proteins.

2.4. Cell lines, transfections and reporter gene assay

Mouse fibroblast NIH3T3 and mouse EC F9 cell lines were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The transient transfection experiments were carried out using Lipofectamine and Plus reagent (Gibco BRL) according to the manufacturer's instructions. Cell extracts were prepared and assayed for luciferase (LUC) activity, according to the manufacturer's instructions (Promega), using the luminometer Berthold LB9506. Total protein quantification in the extracts was determined by the Bradford assay and LUC activity of equal amount of proteins was determined and normalized for β -galactosidase activity.

2.5. Chromatin immunoprecipitation assay (ChIP)

Chromatin immunoprecipitation [35,36] was performed using the ChIP assay kit (Upstate Biotechnology). Approximately 2 million cells transfected (cell transfection efficiency was about 60%) with pRK5-Blues were cross-linked with 1% formaldehyde, for 10 min, at 37°C and lysed. The cell lysate was sonicated on ice, resulting in a DNA fragment of length approximately 500 bp. Immunoprecipitation was performed overnight, with 5 μ g of anti-HA antibody. DNA representing 0.005% of the sonicated chromatin solution (input) and 10% of the immunoprecipitated sonicated chromatin solution were amplified using murine FGF-4 enhancer-specific primers (FGF-4enhF 5'-CTGGGAGACTTCTGAGCAACCTCC-3' and FGF-4enhR 5'-ATTGTCCTGTGAGCCACCAGACAG-3'). Polymerase chain reaction (PCR) conditions were: 95°C for 5 min, followed by 40 cycles at 94°C for 45 s, 62°C for 30 s, 72°C for 30 s.

3. Results

3.1. Structure of the Blues gene

The Blues gene was constructed to produce a protein (pBlues) with the highest probability of targeting the DNA sequence: 5'-GTT-TGG-ATG-3', that we named **Blues target** sequences (BTS) (Fig. 1A). BTS is present in the 3' enhancer region of the mouse FGF-4 gene [24] (Fig. 1B). The Blues gene is 282 bp long, encoding three zinc finger domains (Fig. 1A). The zinc finger backbone used to construct Blues was related to both the Zif268 natural gene product [33] and the Jazz artificial gene product [14]. The pBlues DNA binding capacity was specified on the basis of the code signatures. Each finger domain appears to behave as an independent DNA binding module, making contacts with three adjacent base pairs on one DNA strand [7]. In order to obtain Blues protein, the second and the third zinc fingers of the Zif268 gene have been modified at positions -1, +3, +6 of the α -helix [1,7]. The resulting pBlues structure was the following:

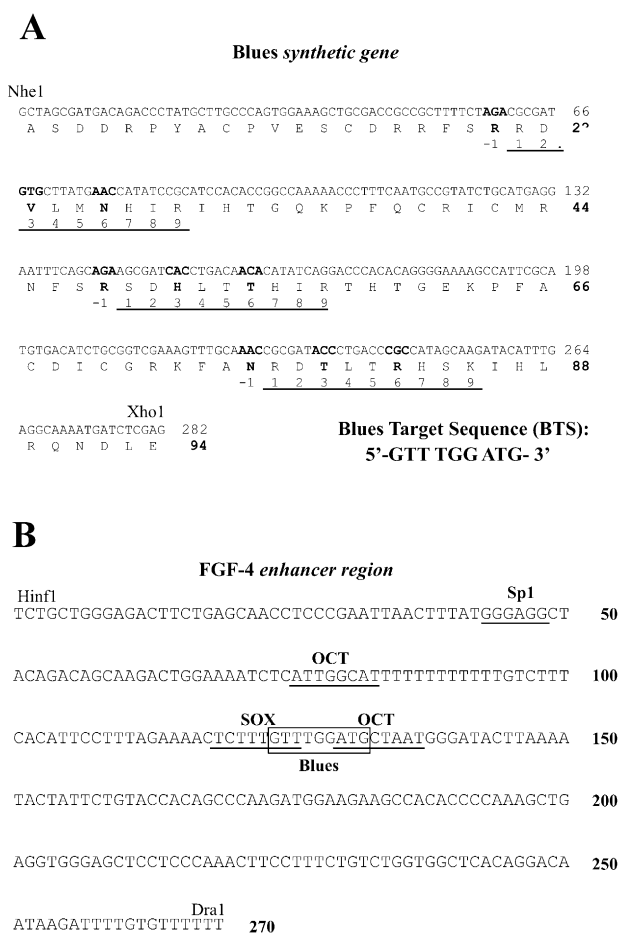


Fig. 1. Schematic representation of Blues and its DNA target. A: Nucleotide and amino acid sequences of the synthetic three zinc finger gene Blues (accession number AY364163). Amino acid residues used in the critical positions of the α -helix are indicated in bold characters. The amino acid stretches composing the α -helix regions present in each finger domain are numbered and underlined. The putative Blues DNA target sequence (BTS) is indicated in bold characters. B: Nucleotide sequence of the murine FGF-4 3' enhancer region. Main transcription factor binding sites are underlined. The Blues target sequence is boxed and partially overlaps SOX and OCT binding sites.

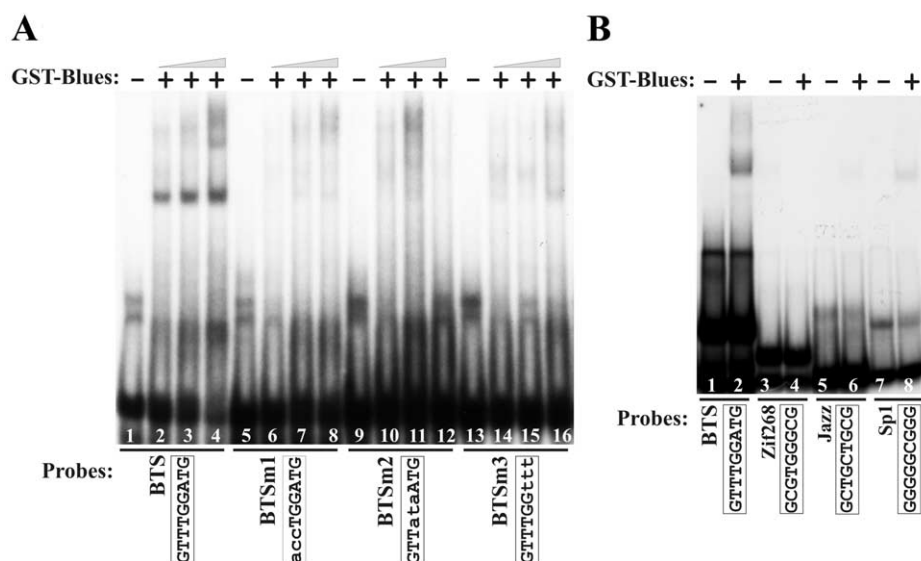


Fig. 2. DNA binding specificity of GST-Blues protein. A: Contribution to DNA binding activity of each Blues zinc finger domain analyzed by EMSA. Increasing amounts of GST-Blues protein were incubated with equal amount of labeled BTS (lanes 1–4) and with labeled mutant targets (lanes 5–16). The mutated triplets in each mutant target are indicated in lowercase. B: EMSA performed with equal amount of following labeled probes: BTS (lanes 1, 2), Zif268 (lanes 3, 4), Jazz (lanes 5, 6) and Sp1 (lanes 7, 8).

the third finger domain, at the carboxy-terminal region, was constructed to recognize the DNA triplet 5'-GTT-3', the second finger recognized the triplet 5'-TGG-3', finally the first finger recognized the triplet 5'-ATG-3' (Fig. 1A).

3.2. GST-Blues DNA binding properties

To test the binding properties of pBlues toward its putative DNA target sequence, we performed EMSAs using the fusion protein GST-Blues, produced and purified in *Escherichia coli*. Fig. 2A shows an evident shift of the oligonucleotide probe containing the double-stranded BTS target in the presence of increasing amounts of GST-Blues (lane 1–4). To obtain a more subtle definition of the contribution of each Blues finger to DNA binding specificity, oligonucleotide target mutants,

with only one of the DNA triplets changed (lanes 5–8, 9–12, 13–16 respectively), were assayed in the same EMSA. As shown in Fig. 2A, GST-Blues binds the BTS probe with affinity and specificity strikingly higher than the BTS mutants: BTSm1, BTSm2 and BTSm3. In the presence of BTSm1 and BTSm3 probes, whose mutation affects the first and third triplet respectively, pBlues DNA binding is dramatically compromised, while in the presence of BTSm2 the shift is completely lost. This is consistent with the crucial role of the target central DNA triplet in the binding of a three zinc finger protein. Moreover, to further confirm the pBlues specificity toward BTS, we have assayed GST-Blues in EMSA using as probe other DNA target sequences recognized by both natural zinc finger proteins (Sp1 and Zif268) and the artificial zinc

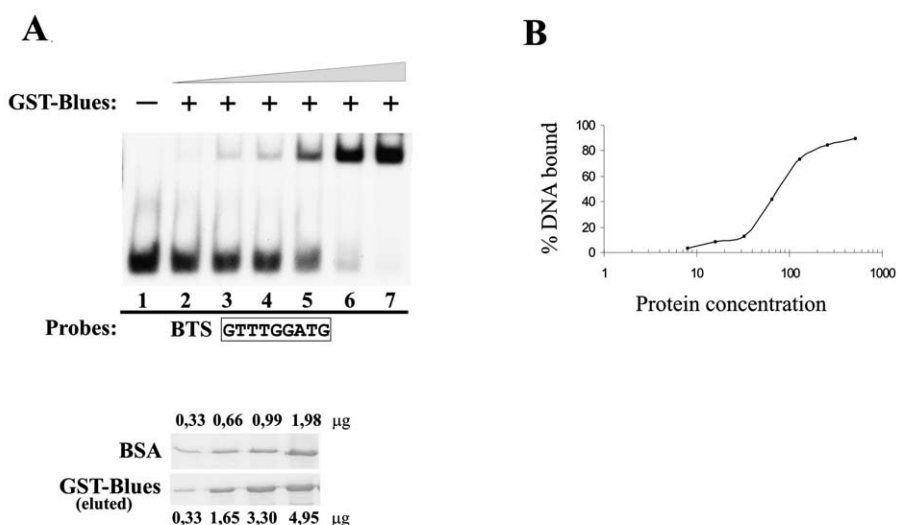


Fig. 3. Determination of the dissociation constant (K_d) by EMSA. A: Lanes 1–7 show: 0, 8, 16, 32, 64, 128 and 256 nM of GST-Blues protein incubated with labeled BTS (top panel); quality control of purified/eluted GST-Blues protein by Coomassie staining, compared with purified commercial bovine serum albumin (bottom panel). B: EMSA-derived curve. The protein concentration is expressed in nM.

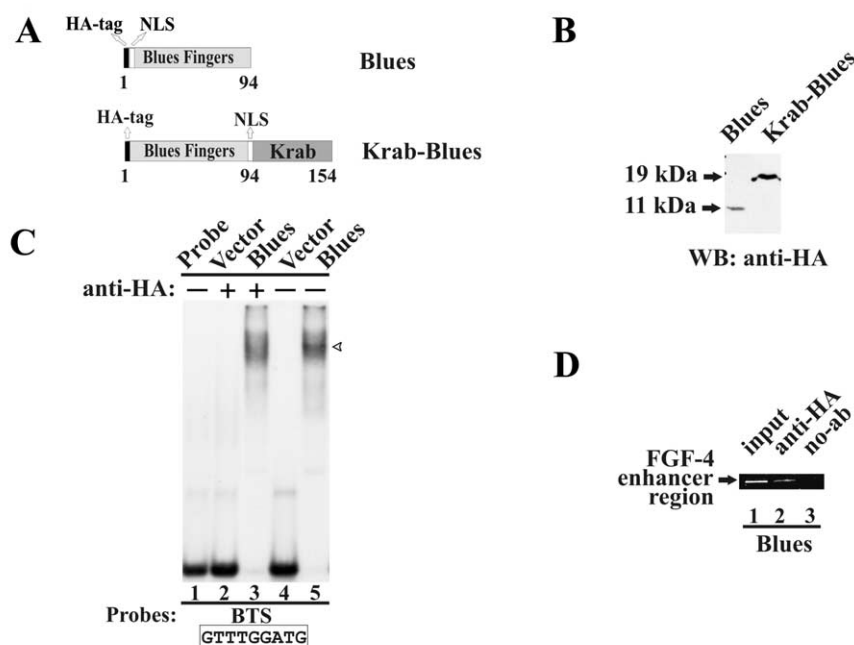


Fig. 4. DNA binding activity of pBlues in mammalian cell lines. A: Schematic representation of the chimeric Blues and Krab-Blues proteins. The SV40 nuclear localization signal (NLS) and the HA epitope are indicated. B: Western blot analysis of Blues and Krab-Blues proteins, prepared from total extracts of transiently transfected NIH3T3 cells and revealed using 12CA5 monoclonal antibody. C: EMSA of pBlues expressed in NIH3T3 cells. D: pBlues chromatin immunoprecipitation, performed in transfected F9 cells, using either 12CA5 monoclonal antibody/protein A-agarose beads or only protein A-agarose beads as a control (no-ab). Immunoprecipitates from each sample were analyzed by PCR. A sample representing linear amplification of the total input chromatin (input) was included in the PCR as a control (lane 1).

finger peptide (Jazz) [14]. As shown in Fig. 2B, in the presence of GST-Blues (lane 2), the signal of BTS shift is strongly higher than those obtained with both Jazz and Sp1 targets (lanes 6 and 8), while with Zif268 target the shift is almost undetectable (lane 4). To measure the affinity of the GST-Blues for the BTS target the relative dissociation constant (K_d) was determined by EMSA performed using increasing amounts of GST-Blues, combined with a constant amount of labeled BTS (Fig. 3). In our experimental conditions, we obtained a K_d of about 75 nM. These data strongly indicate that GST-Blues efficiently binds its 'code-programmed' target.

3.3. Blues expression and DNA binding activity in eukaryotic cell lines

In order to characterize the biological activity of pBlues, we expressed the synthetic protein in mammalian cell lines both as a simple three finger peptide and as a fusion with the transcriptional repression domain Krab (Krab-Blues) (Fig. 4A). The resulting molecules (pBlues and Krab-Blues) were expressed in the NIH3T3 cell line as HA-tagged proteins, under the control of the regulative regions derived from cytomegalovirus. By Western blot analysis we checked the expression and the correct size of Blues and Krab-Blues proteins (Fig. 4B). To study the DNA binding properties of pBlues, EMSAs were performed using cell nuclear extracts derived from NIH3T3 transiently transfected with the construct expressing pBlues. In Fig. 4C, the protein present in the extracts bound to BTS is indicated by an arrowhead (lane 5). The identity of the protein complexed with BTS was further confirmed by both the presence of a pBlues supershift and a partial disappearance of pBlues shift, when the HA tag antibody was added to the reaction mixture (lane 3).

3.4. Chromatin immunoprecipitation of Blues

The effectiveness of pBlues in specific DNA binding led us to employ a more direct strategy to investigate the accessibility of pBlues to the chromatin infrastructure. To this end, a ChIP assay was performed in F9 cells. The Blues expression

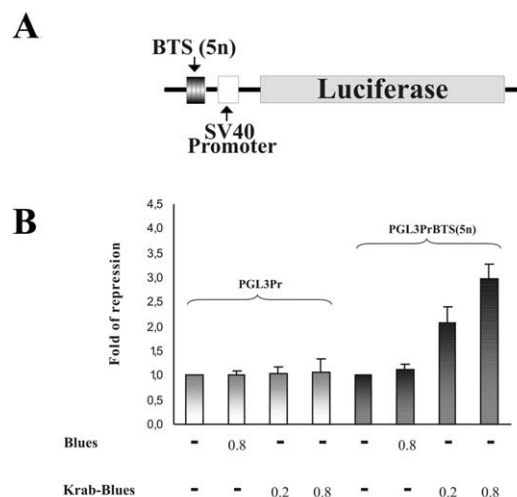


Fig. 5. Trans-repression of pGL3PrBTS(5n) construct by Krab-Blues and Blues proteins. A: Schematic representation of the LUC construct containing five copies of the BTS, upstream of the SV40 promoter. B: Histograms show fold repression of the pGL3PrBTS(5n) or pGL3Pr reporter constructs (0.1 µg), obtained upon co-transfection with either Blues (0.8 µg) or Krab-Blues (0.2 µg and 0.8 µg) expression vectors in NIH3T3 cells. The data are presented as the means ± S.D. of three independent transient transfection experiments performed in duplicate.

construct was transiently transfected and after cross-linking and immunoprecipitation with specific antibody, the FGF-4 enhancer, containing the BTS sequence, was PCR-amplified. As shown in Fig. 4D pBlues is able to bind its target sequence at the endogenous chromosomal site.

3.5. Blues transcriptional activity

The next step was to test whether Krab-Blues chimeric protein was able to modulate the expression of a test gene driven by the region of FGF-4 enhancer responsible for Blues binding. To this end Blues and Krab-Blues expression constructs were separately transfected into NIH3T3 cells with the LUC reporter construct pGL3PrBTS(5n) (Fig. 5A). As shown in Fig. 5B, Krab-Blues represses the LUC activity of pGL3PrBTS(5n), by about two- to three-fold in a dose-dependent manner. As expected, the co-transfection of pGL3PrBTS(5n) with Blues containing only the zinc finger region as well as the co-transfection of the control vector (pGL3Pr) with either Blues or Krab-Blues did not give any LUC repression activity. These results, together with the DNA binding data reported above, strongly support the notion that Krab-Blues chimeric protein is able to repress transcription from a test gene through specific binding to the 9 bp BTS present in the murine FGF-4 enhancer.

4. Discussion

The possibility of re-programming the expression of specific genes represents a promising tool in basic science, biotechnology and medicine. In particular, disease-related genes can be either turned off or enhanced for the purpose of treating conditions such as cancer or viral infection. An important technology for achieving this goal is the rational design of zinc fingers based on the recognition code. Using the available list of the amino acid–base contacts dictated by the recognition code, we have realized several synthetic zinc finger genes [12–14]. Here we report the design, construction and functional characterization of the novel artificial gene, Blues, whose product is able to bind and to repress transcription from the 3' enhancer of the FGF-4 gene. Using different approaches, we have shown that pBlues is able to recognize its DNA target. The complex pBlues/target is stable and its K_d is comparable to the values obtained for other artificial and natural zinc finger proteins [12–14,37,38]. The calibration of the correct degree of DNA binding affinity and specificity is one of the crucial matters in designing and selecting synthetic zinc finger peptides [7,16]. We demonstrated that pBlues recognizes its 9 bp programmed DNA target with a proper balance between affinity and specificity. Statistically assuming random base distribution, a 9 bp long DNA target sequence should be present in the human genome ($\sim 3.5 \times 10^9$ bp) about 1.3×10^4 times. Therefore, for a target sequence to be unique in the human genome requires a length at least 16 bp [14]. Of course, since the chromatin infrastructure protects most of the genome from DNA binding proteins, only a small portion of their potential sites is accessible, so that the target length required for specific recognition in the chromosomal context can be much shorter. Significantly, the genomic locus of the FGF-4 enhancer, containing the Blues target sequence, includes the binding sites of two other well-characterized transcription factors, Sox-2 and Oct-3, suggesting for this region an accessible conformation of the chromatin. By ChIP experi-

ments, we confirmed the effective access of pBlues to active chromatin of the FGF-4 enhancer region. This last result, together with the transcriptional data, is particularly promising in view of conducting an in vivo study of the effect on transcription of FGF-4 growth factor, regulated by a Blues module fused to chosen regulatory domains. The ability to modulate the expression of the FGF-4 gene at will represents a powerful tool to shed light on the role of FGF-4 in tumorigenesis/angiogenesis and on its multiple role during development. Furthermore, re-programmed expression of FGF-4 could have tremendous therapeutic and biological applications.

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