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Mutations in the basic domain and the loop–helix II junction of TWIST abolish DNA binding in Saethre–Chotzen syndrome

Vincent El Ghouzzi, Laurence Legeai-Mallet, Catherine Benoist-Lasselin, Elisabeth Lajeunie, Dominique Renier, Arnold Munnich, Jacky Bonaventure*

Unité de Recherches sur les Handicaps Génétiques de l'Enfant, INSERM U-393, Institut Necker, 149 rue de Sèvres, Paris, France

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Abstract Saethre–Chotzen syndrome is an autosomal dominant skull disorder resulting from premature fusion of coronal sutures (craniosynostosis). It is caused by mutations in the *TWIST* gene encoding a basic Helix–Loop–Helix transcription factor. Here we report on the identification of a novel mutation affecting a highly conserved residue of the basic domain. Unlike nonsense and missense mutations lying within helices, this mutation does not affect protein stability or heterodimerisation of TWIST with its partner E12. However, it does abolish TWIST binding capacity to a target E-box as efficiently as two missense mutations in the loop–helix II junction. By contrast, elongation of the loop through a 7 amino acid insertion appears not to hamper binding to the DNA target. We conclude that loss of TWIST protein function in Saethre–Chotzen patients can occur at three different levels, namely protein stability, dimerisation, and DNA binding and that the loop–helix II junction is essential for effective protein–DNA interaction.

Key words: Saethre–Chotzen syndrome; Craniosynostosis; *TWIST* gene; bHLH transcription factor; DNA binding

1. Introduction

Saethre–Chotzen syndrome (SCS; MIM 101400) is an autosomal dominant craniosynostosis syndrome characterised by the premature fusion of coronal sutures leading to skull deformation, accompanied by limb abnormalities of variable severity and facial dysmorphism. This disorder has been ascribed to mutations in the human *TWIST* gene (*H-TWIST*) which encodes a class B basic Helix–Loop–Helix (bHLH) transcription factor [1,2]. *Twist* was first identified in *Drosophila* (*D-Twist*) as a zygotic developmental gene involved in early mesoderm patterning [3,4]. Subsequently, several orthologs of *D-Twist* have been identified in vertebrates: *Xenopus* (*X-Twist*, [5]), mouse (*M-Twist*, [6]), and human (*H-TWIST*, [7,8]), as well as a *Twist*-related factor in *Caenorhabditis elegans* (*Ce-Twist*, [9]). Moreover, there is a high degree of homology in the bHLH domain; i.e. 84% amino acid identity between *Drosophila* and vertebrates and 94–100% among vertebrates. Twist does not seem to be critical for mesoderm formation in vertebrates as *Twist*−/− mice develop mesoderm but die at embryonic day 11.5 [10]. However, *Twist* is required for the proper regulation of muscle development in both invertebrates [9,11] and vertebrates [12–15] suggesting that some of its developmental functions and signalling pathways could have been conserved during evolution.

The identification of mutations in *H-TWIST* as the underlying cause of SCS has uncovered a novel function for this gene in the regulation of membranous bone ossification [1,2,16]. So far, more than 50 different mutations scattered over the coding sequence of *H-TWIST* have been reported in SCS (for mutation update see [17]), most of which either create premature termination codons or substitute highly conserved residues in the bHLH region [18]. To date, however, no obvious genotype–phenotype correlation has been observed in SCS. Observations based on complete heterozygous deletions of *H-TWIST* in some SCS patients and on the phenotype of *Twist*+/− mouse suggest haploinsufficiency as the most likely disease-causing mechanism [1,16,19]. In support of this, we recently showed that amino acid changes in either helix I or II result in mutant proteins that fail to dimerise with the class A bHLH factor E12 (one of the two products of the *E2A* gene, [20]) and become abnormally localised in the cytoplasm [21]. By contrast, mutations altering the loop domain had mild or no effect on either protein dimerisation or its cellular localisation. As patients harbouring *TWIST* mutations in either helix or loop domains are clinically indistinguishable, we hypothesised that mutations in the loop could result in haploinsufficiency via another molecular mechanism.

In this study, we tested the binding capacity of the TWIST/E12 complex in several mutant proteins arising from either an in frame insertion lying in the middle of the loop (P139ins7), or two missense mutations in the loop–helix II junction (S144R and K145E), or a novel mutation in the basic domain of H-TWIST (R118C), to a target E-box. While R118C, S144R and K145E mutant proteins displayed normal stability and were able to shuttle to the nucleus as heterodimers with E12, they failed to interact with the DNA binding site. By contrast, the elongated protein resulting from the loop insertion was still able to dimerise and bind the target E-box.

2. Materials and methods

2.1. DNA analysis

Blood samples were obtained with the written consent of the patients and genomic DNA was extracted from leukocytes according to standard procedures. The whole coding sequence of the human *TWIST* gene was amplified as previously described [18] and amplifi-

*Corresponding author. Fax: (33)-1-47 34 85 14.
E-mail: bonavent@necker.fr

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cation products were sequenced by using the fluorescent dideoxy terminator method on an automatic sequencer ABI 373.

2.2. Expression vectors and in vitro protein–protein interaction assay

For eukaryotic expression in Cos7 cells and in vitro synthesis of TWIST proteins, the whole coding sequence of *TWIST* was directly PCR-amplified from genomic DNA of control and SCS patients using the following primers: Twi-*Bam*HI (forward) 5′-GACAGGATCCGGAGGAATGATGCAGGACGTGTCCAG-3′ and Twi-*Sal*I (reverse) 5′-GACTGTCGACCTAGTGGGACGCGGACATGG-3′. Mutant alleles were cloned into either the pCR3 unidirectional plasmid or the pcDNA3.1/*myc*-His plasmid (Invitrogen, San Diego, CA, USA). Clones were sequenced and confirmed to be free of errors. For in vitro protein–protein interaction assays, wild-type, R118C, S144R, K145E and P139ins7 *TWIST* cDNAs were transcribed and translated from pCR3uni constructs using the TNT T7 MasterQuick transcription/translation system (Promega, Madison, WI, USA) in the presence of [^{35}S]methionine (1000 Ci/mmol; Amersham Pharmacia Biotech, UK). Glutathione *S*-transferase (GST)–E12 fusion proteins were produced in BL21 *Escherichia coli* cells transformed with the pGEX4T-1/E12wt construct and interaction assays were performed as previously described [21].

2.3. Cell culture, transfection and immunofluorescence

Cos7 cells were seeded in either 6-well tissue culture plates or 8-well chamber slides with 1.5×10^5 and 1×10^4 cells/well respectively, and cultured to 70% confluency. Transfections were performed with Fugene-6 (Roche Molecular Biochemicals, Uppsala, Sweden) and 2 or 0.1 μg DNA/well according to the manufacturer's instructions. Transfected cells in chamber slides were fixed 24 h later with 4% paraformaldehyde for 30 min. After permeabilisation with 0.1% Triton X-100 for 15 min, cells were incubated for 1 h at room temperature with a goat polyclonal anti-TWIST antibody at dilution 1:100 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Cells were then incubated with a fluorescein isothiocyanate (FITC)-conjugated donkey anti-goat antibody and examined with a Zeiss LSM 510 confocal microscope.

2.4. Electrophoretic mobility shift assays (EMSAs)

10 μg of total Cos7 lysate were used per binding reaction (total volume of 15 μl) and incubated with a ^{32}P-labelled synthetic double-strand oligonucleotide containing a *Nde*I E-box adapted from [22] (5′-CCCCCACCATATGGTGCCTGA-3′). Binding reactions were carried out in a buffer containing 80 mM magnesium acetate, 200 ng/μl poly dIdC, 1 μg/μl ssDNA, 10 mM NaH_2PO_4/Na_2HPO_4, 1 mM EDTA, pH 7.2, incubated on ice for 15 min and samples were then electrophoresed at room temperature on native 5% polyacrylamide/0.5×TBE gels. For supershift assays, 5–10 μg anti-E12 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-Myc antibodies (Roche Molecular Biochemicals, Uppsala, Sweden) were added to the binding reaction. Gels were fixed for 15 min in 10% acetic acid/10% methanol solution, dried under vacuum for 45 min and exposed to films (Biomax RM, Eastman Kodak Company, Rochester, NY, USA) in the dark at −80°C for time periods between 6 h and 2 days.

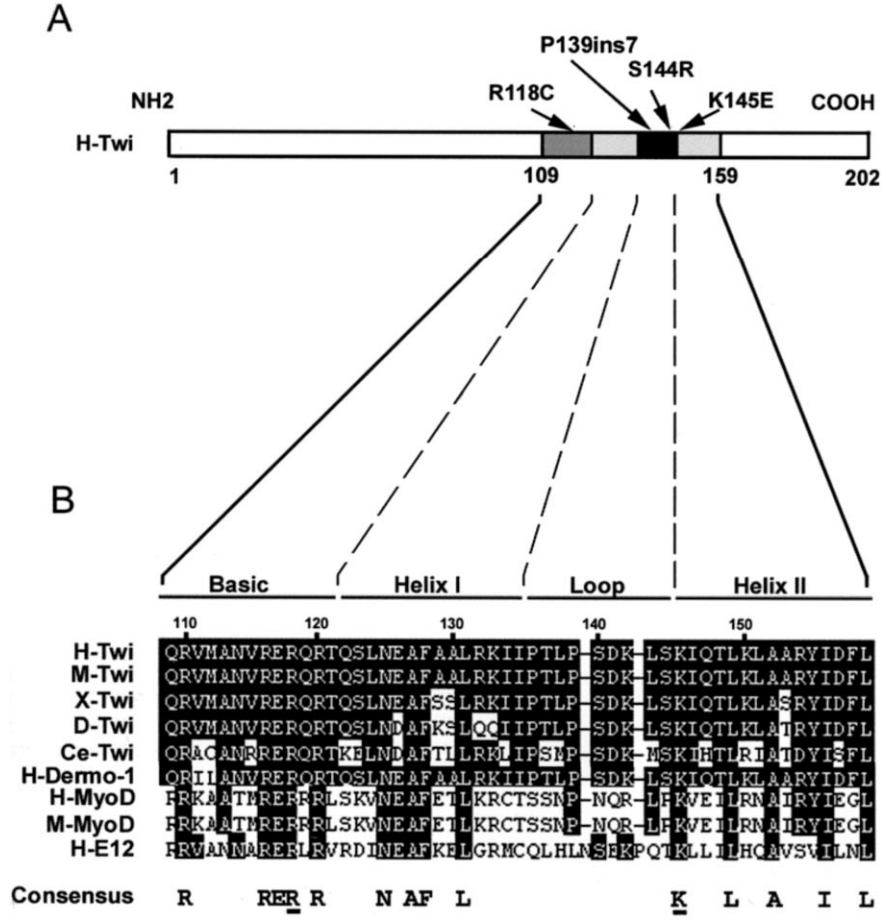

Fig. 1. Location of four SCS mutations affecting the bHLH domain of the TWIST protein. A: Schematic representation of the human TWIST protein showing positions of the R118C, P139ins7 (in frame duplication of KIIPTLP), S144R and K145E mutations in the basic domain, the middle of the loop, the end of the loop and the start of helix II, respectively. B: Multiple sequence alignments of the bHLH region from different Twist orthologs ((H), Human; (M), Mouse; (X), *Xenopus*; (D), *Drosophila*; (Ce), *C. elegans*) and from Dermo-1, MyoD and E12. Sequences were aligned using the CLUSTAL-W program [35]. Conserved residues are shown on a black background and mentioned in the consensus sequence. Note that two mutant residues (arginine 118 and lysine 145, underlined in the consensus sequence) are highly conserved through evolution.

3. Results

3.1. Identification of a novel SCS mutation in the basic domain of H-TWIST

Clinical and radiological examination of a man and his two affected sons revealed typical features of SCS, namely, unilateral or bilateral coronal suture fusion, small ears with crura and low frontal hair line. Direct sequence analysis of the coding region of *TWIST* revealed a C to G transition at position 352 segregating with the disease and causing an arginine to cysteine substitution in the basic domain (R118C). The location of this amino acid change and that of three previously reported mutations [18] affecting the centre of the loop (P139ins7) and the loop–helix II junction (S144R, K145E) are presented in Fig. 1A. Alignment of bHLH amino acid sequences from other transcription factors revealed that arginine 118 and lysine 145 are highly conserved both across species and among members of the bHLH transcription factor family, while serine 144 is less conserved (Fig. 1B).

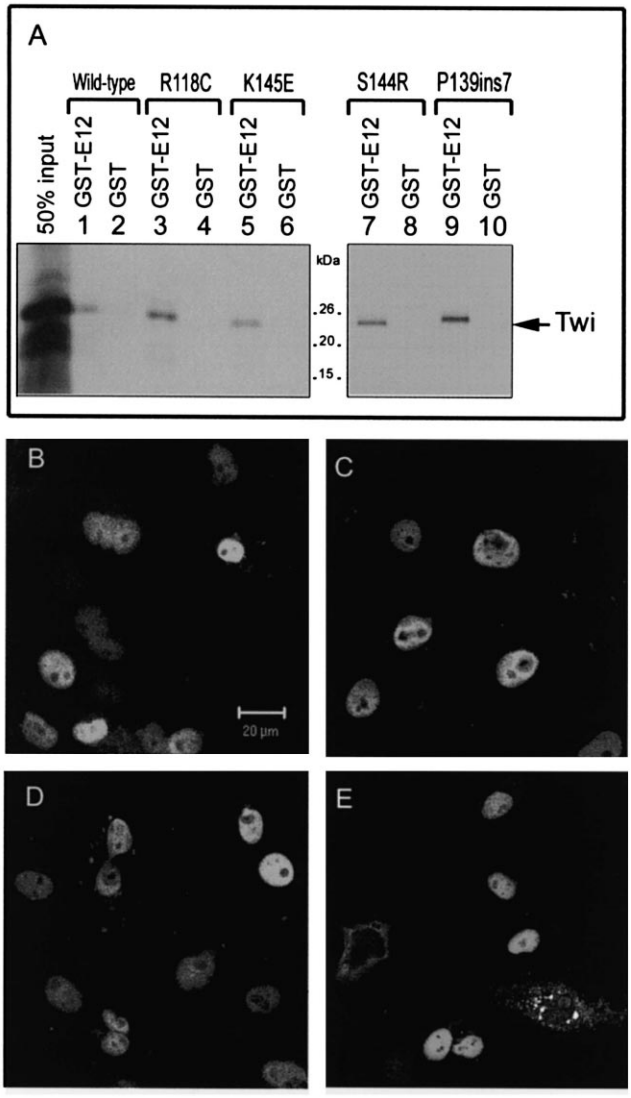

Fig. 2. Dimerisation capacities and subcellular location of wild-type and mutant TWIST proteins. A: In vitro interaction between ^{35}S-labelled wild-type TWIST and GST–E12 (lane 1) was not affected by the R118C, K145E, S144R or P139ins7 mutations (lanes 3, 5, 7 and 9, respectively). As expected, no interaction occurred between TWIST proteins and GST alone (lanes 2, 4, 6, 8 and 10). Half the amount of radiolabelled TWIST protein used for the assay is shown as an input. B–D: Subcellular immunolocalisation of the wild-type and mutant TWIST proteins in Cos7 cells co-transfected with E12. Mutant proteins in the basic domain (C) and the loop–helix II junction (D) disclosed the same nuclear staining as the control (B). Insertion in the loop induced both cytoplasmic and nuclear localisation of the elongated protein (E).

3.2. Expression, dimer formation and intracellular localisation of basic and loop mutant proteins

To determine how mutations of the basic and loop domains alter protein function in SCS, human *TWIST* cDNAs carrying the R118C, the S144R, the K145E or the P139ins7 genotypes were cloned in an expression vector, translated in vitro in a reticulocyte lysate system and found to result in molecules of the expected size (data not shown). RNA and protein stability was then tested by ectopically expressing wild-type and mutant constructs in transfected Cos7 cells. Northern blot and immunoblot analyses of cell lysates showed that, 24 or 48 h post-transfection, the amount of mutant transcripts and proteins were similar to controls, suggesting that mRNA and protein stability remained unaltered (not shown).

The mutant proteins were still able to interact with E12 fused to GST (GST–E12), as shown by the binding of in-vitro-translated TWIST proteins to the GST–E12 fusion protein (Fig. 2A). Additional evidence of these interactions stems from yeast two-hybrid assays that revealed normal heterodimerisation of the three missense mutants and reduced heterodimerisation of the loop insertion (data not shown, [21]). We conclude that mutations altering either the basic domain or the loop–helix II junction of TWIST do not affect the dimerisation capacities of the protein. Mutant proteins were then co-expressed with E12 in Cos7 cells and their localisation was analysed by confocal immunofluorescence microscopy. Cells transfected with the R118C, S144R or K145E mutant constructs exhibited a strong nuclear staining with anti-TWIST antibodies in a pattern similar to wild-type transfected cells (Fig. 2B–D). Transfection with the P139ins7 mutant induced preferential nuclear localisation but cytoplasmic staining was detected in 30% of transfected cells (Fig. 2E).

3.3. Binding of wild-type TWIST proteins to a DNA sequence specific of the human TWIST/E12 heterodimers

Based on functional data obtained in invertebrates [9,11], and more recently in mouse [23], a putative Twist binding site has been identified. We therefore designed a double-stranded DNA probe containing an hexanucleotide motif CATATG (*Nde*I E-box) for assaying the binding capacities of the human protein. EMSAs were performed using lysates from Cos7 cells expressing H-TWIST, E12 or both proteins. We found that H-TWIST was able to bind the *Nde*I E-box, only when co-expressed with E12 in transfected cells (Fig. 3A, lane 5). The specificity of binding was demonstrated by using: (i) variable amounts of unlabelled probe (Fig. 3A, lanes 6 and 7) that competed with the labelled probe, and, (ii) human anti-E12 antibodies that produced a supershift of the DNA–protein complex (Fig. 3A, lane 8). Since antibodies raised against the amino-terminal domain of H-TWIST gave only a weak supershift (not shown) that could be ascribed to the low affinity of the antibody, we generated a myc-tagged *TWIST* cDNA construct (mtTwi) for co-transfection with E12. Incubation of the cell lysate with an anti-myc antibody resulted in

a supershift of the DNA–protein complex (Fig. 3A, lane 10). Similarly, incubation of the TWIST/E12 complex with a CAGCTG motif (*Pvu*II E-box) known to give no bandshift with the D-Twist protein [11], also failed to produce a bandshift (not shown). Hence, the human TWIST/E12 complex is able to bind the *Nde*I probe and to specifically recognise the CATATG motif. In addition, in vivo heterodimerisation of the TWIST protein with E12 in co-transfected cells appears

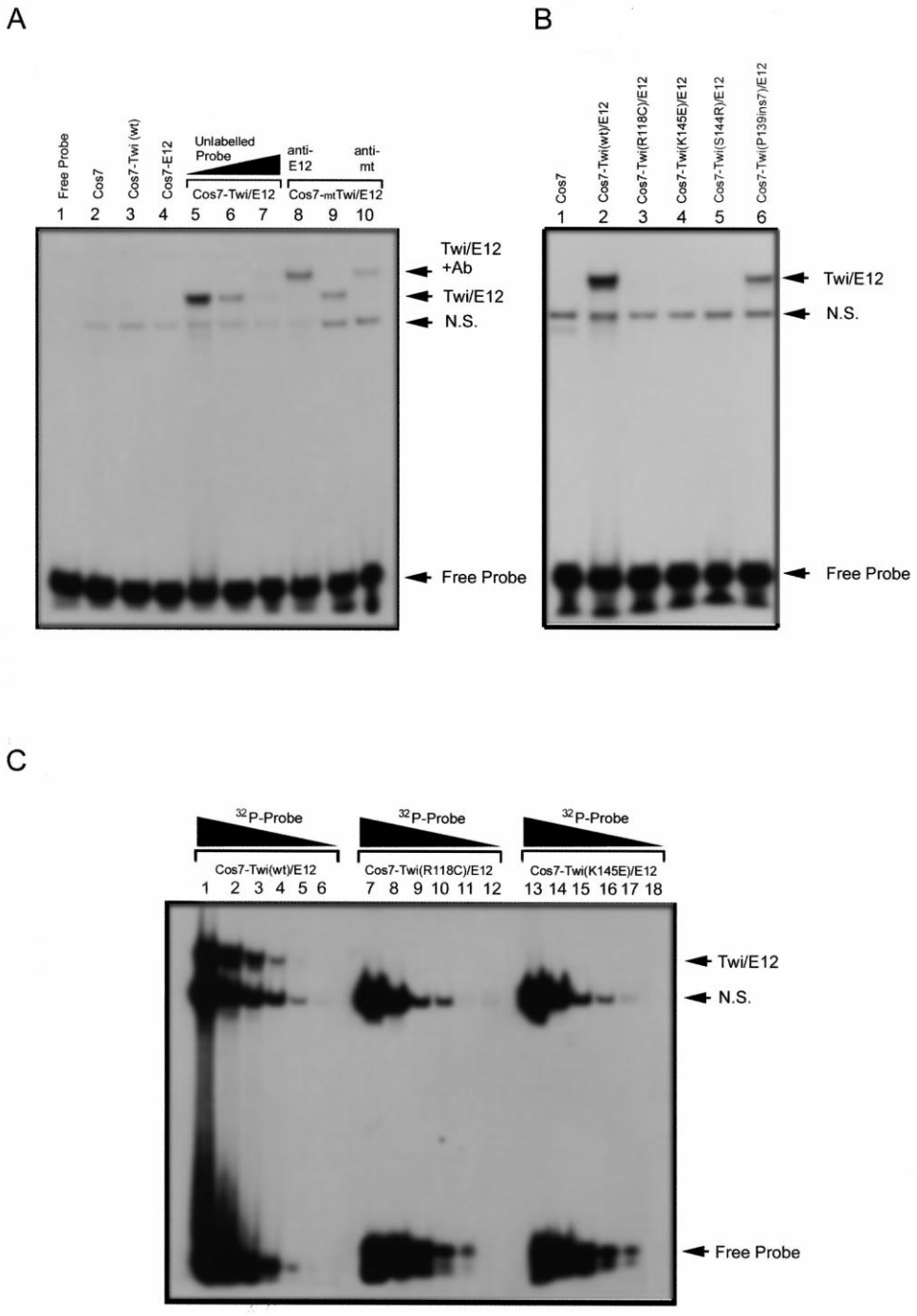

Fig. 3. Absence of TWIST/E12 DNA binding in R118C, S144R and K145E TWIST mutants. A: EMSA of the human TWIST protein to the *Nde*I E-box. Total lysates from untransfected (lane 2) or transfected Cos7 cells (lanes 3–10) were incubated with the radiolabelled probe (6000 cpm). Transfection with either wild-type (wt) TWIST or E12 cDNA constructs gave no specific bandshift (lanes 3 and 4), while co-transfection with TWIST and E12 gave a specific shift (lanes 5 and 9). Unlabelled probe (0.5 and 1.0 nmol) was used to compete for the binding (lanes 6 and 7, respectively). Specificity of the TWIST/E12 complex was assessed by using antibodies against either E12 (lane 8) or myc-tagged TWIST (lane 10). B: EMSA of the wild-type (wt) and mutant TWIST proteins to the *Nde*I E-box (6000 cpm). TWIST/E12 DNA binding (lane 2) is abolished when the R118C, K145E or S144R missense mutations are introduced (lanes 3, 4 and 5, respectively), but persists with the P139ins7 mutation (lane 6). C: EMSA of the wild-type (wt) and mutant TWIST proteins (R118C and K145E) to the *Nde*I E-box using decreasing amounts of the radiolabelled probe, ranging from 260 000 to 1000 cpm (lanes 1–6, 7–12 and 13–18). N.S. = non specific signal, Ab = antibody, mt = myc-tag.

to be required for efficient binding to the CATATG motif, as in vitro co-incubation of cell lysates derived from Cos7 cells separately transfected with either TWIST or E12 resulted only in a weak bandshift (not shown).

3.4. Mutations in the basic domain and the loop–helix II junction abolish TWIST DNA binding activity

The normal stability of basic, loop and loop–helix II mutant proteins and their capacity to form heterodimers led us to hypothesise that in these cases, the loss of TWIST function could result from an impairment of protein–DNA interaction. Therefore, we performed EMSA by using the *Nde*I E-box as a probe and Cos7 lysates expressing E12 and R118C, S144R, K145E or P139ins7 TWIST mutant proteins. Both basic and loop–helix II junction missense mutations induced a loss of the TWIST/E12 DNA binding activity, while insertion in the loop did not hamper the binding (Fig. 3B). To test whether the absence of bandshift resulted from a complete loss of the binding activity or a decreased affinity of the mutated complex for the probe, we repeated the experiment using variable amounts of radiolabelled *Nde*I probe. Fig. 3C shows that both R118C and K145E TWIST/E12 complexes failed to give a bandshift even when high amounts of probe were used, indicating that both mutations abolished TWIST–DNA binding activity. Similar results were obtained with the S144R mutant protein (not shown).

4. Discussion

Although SCS mutations commonly involve the bHLH region of H-TWIST [17,18], the resulting alteration in gene function depends on the particular protein subdomain affected. Nonsense mutations upstream or within the bHLH motif cause the synthesis of truncated proteins which are rapidly degraded [21], while missense mutations involving helix I or II region create proteins that fail to heterodimerise and become abnormally located in the cytoplasm [21]. Surprisingly, missense mutations in the loop–helix II junction of the bHLH motif affect neither protein stability nor the ability to form heterodimers. Hence, the goal of the present study was to explore alternative mechanism(s) by which mutations in the basic or loop domains of TWIST alter protein function in SCS. Using an assay that relies on the high binding affinity of the TWIST/E12 heterodimer for the *Nde*I E-box, we show here that unlike in frame insertion in the loop, missense mutations in either the basic domain or the loop–helix II junction of H-TWIST yield proteins that lack *Nde*I E-box binding capacity. Our results therefore identify a novel mechanism by which a Twist mutation can result in haploinsufficiency in SCS (Table 1).

Table 1
Summary of the different mechanisms accounting for haploinsufficiency in SCS

	Stability	Dimerisation	Nuclear location	DNA binding
Nonsense	−	−	−	−
Missense				
Basic (B)	+	+	+	−
Helix I (H)	+	−	−	−
Loop–helix II				
Junction (LH II)	+	+	+	−
Helix II (H)	+	−	−	−

TWIST nonsense and missense mutations alter gene function in different ways: (i) nonsense mutation affect protein stability, (ii) missense mutations in helices (H) hamper heterodimerisation with E12 and nuclear location, (iii) missense mutations in the basic domain (B) or at the loop–helix II junction (LH II) abolish DNA binding to the *Nde*I E-box. Affected or unaffected functions are marked with (−) or (+) signs respectively.

bHLH transcription factors are known to bind DNA motives consisting of hexanucleotide sequences CANNTG called E-boxes [24], located in the promoter and enhancer regions of target genes. One such cluster termed *Nde*I E-box, which is known to interact with D-Twist, has been found in the upstream region of the *NK-4*/*Tinman* homeobox gene [11]. Two such palindromic motives have also been found in a 22-bp enhancer element of the *ceh-24* gene which encodes a *C. elegans* NK-2 homeodomain transcription factor [9] and recently, Kophengnavong et al. showed that this same *Nde*I E-box can bind M-Twist, although no target gene has yet been identified in mouse [23]. In their in vitro assays, M-Twist/E12 heterodimers bound DNA more efficiently than M-Twist alone or E12 homodimers. These observations differ from ours, however, in that we could not detect any bandshift for human TWIST in the absence of E12. Moreover, we found that E12 homodimers were also unable to bind the CATATG motif, further supporting the hypothesis that heterodimerisation of H-TWIST with E12 in vivo is essential for transcriptional activity of human TWIST.

The loss of interaction between the basic domain and the CATATG motif as a mechanism can be further explored by three-dimensional analysis of the complex formed between TWIST protein and DNA. Because the crystal structure of TWIST protein is still unsolved, we used MyoD dimer complexed with DNA as a model [25]. This would be valid for two reasons. First, there is a high degree of conservation between TWIST arg118 and lys145 on one hand, and MyoD arg119 and lys146 on the other (Figs. 1 and 4). Second, the fold region of different bHLH proteins also appears to be highly conserved, as is the case for example between MyoD, E47 (another bHLH product of the *E2A* gene) and Max1 (a bHLH-zipper transcription factor) [25–27]. Although MyoD and Twist possess different bp at the centre of their CANNTG consensus binding sites (GC for MyoD and TA for Twist), crystallographic analyses of MyoD and E47 complex have shown that these bases do not directly contact the amino acids of the basic region [25,26]. This suggests that these central bases most likely play a role in functional specificity through differences in the conformation of the basic region/DNA complex. Interestingly, replacement of arginine 119 by a cysteine residue in the MyoD/DNA model abolishes the hydrogen bond that normally exists between the arginine side chain and phosphates of the diester backbone forming the E-box (Fig. 4C,D), further emphasising the requirement for an arginine at that position. It is thus tempting to speculate that replacement of TWIST arginine 118 by a cysteine would similarly disrupt the interaction with the phosphodiester backbone and account for our results. Of particular interest is the observation that in the loop–helix II junction of MyoD, lysine 146 (equivalent to lysine 145 in H-TWIST) is located in the vicinity of the DNA strand (Fig. 4A,B). This lysine donates a hydrogen bond to the phosphate group of adenine forming the E-box through its side chain [25]. Similar conclusions have been drawn from the E47/DNA crystal structure [26]. In the case of Twist, substitution of lysine 145 by

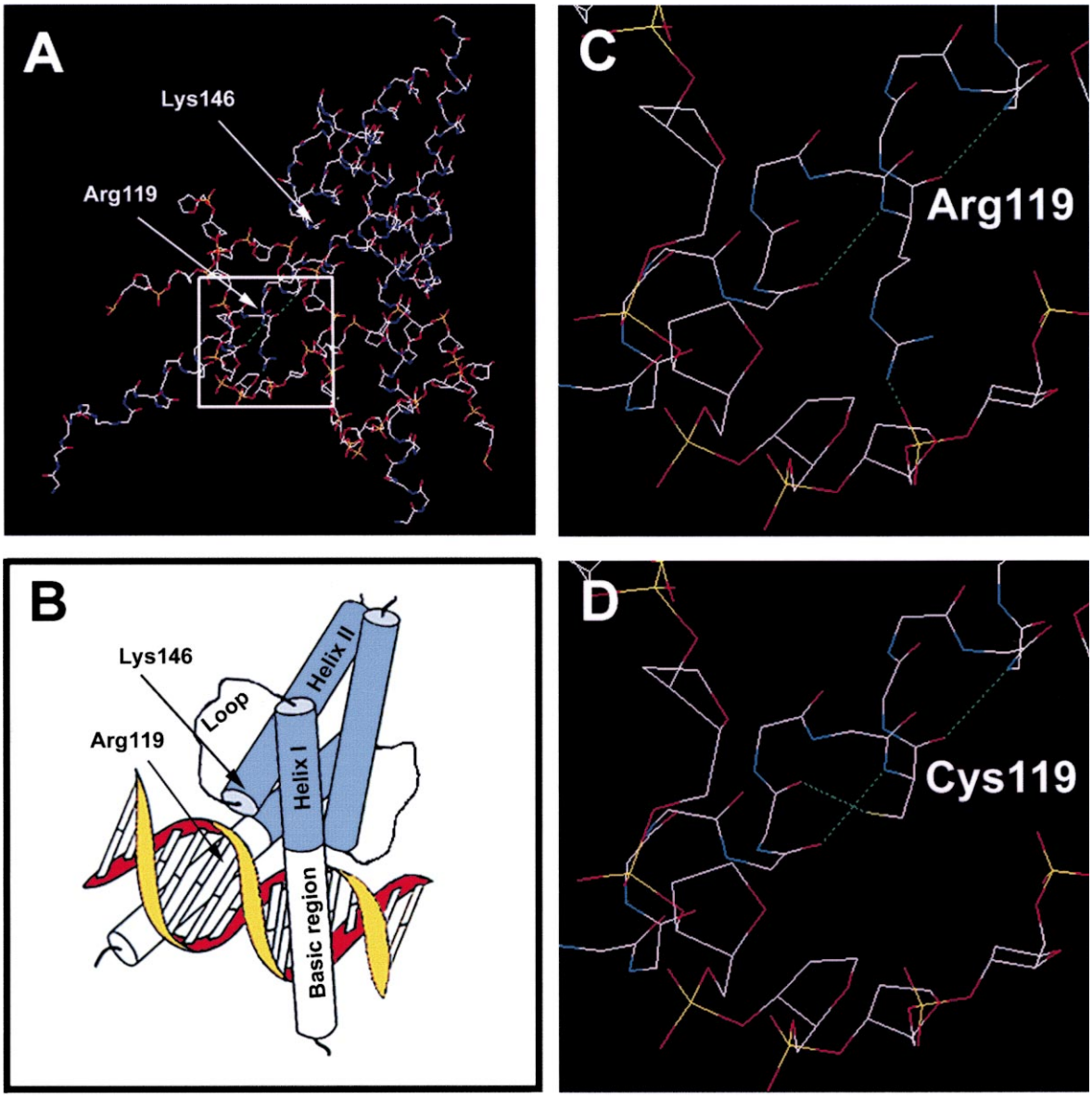

Fig. 4. Three-dimensional interpretation of the consequences of the TWIST R118C mutation on the protein–DNA complex using the MyoD–DNA interaction model. A: Stereodiagram of a MyoD–DNA complex drawn from [24] showing interaction between DNA (in red, yellow and white) and the basic regions of two MyoD monomers (in red, blue and white). Mutated amino acids, equivalent to arg118 and lys145 in the H-TWIST protein, are indicated by arrows. B: Sketch of the protein–DNA complex in the same orientation as in A. The 14-bp DNA site is represented as yellow and red ribbons. The α-helices (I and II) of the bHLH domain are represented in dark blue and the basic regions are in light blue. Location of mutated amino acids is shown by arrows. C: Higher magnification of the inset in A showing the presence of an hydrogen bond (dotted green line) between the lateral chain of arginine 119 (in white and blue) and a phosphate residue (in yellow and red) of the sugar-phosphate DNA backbone. D: Disruption of protein–DNA interaction caused by substitution of arginine 119 by a cysteine residue. The cysteine lateral chain is unable to form an hydrogen bond with a phosphate residue of the DNA backbone. Colour coding is as follows: white, carbon atoms; red, oxygen atoms; blue, nitrogen atoms; yellow, phosphorus atoms. The Swiss-PdbViewer program (v3.51) from GlaxoWellcome Experimental Research was used to visualise the putative effect of the R119C mutation on the murine MyoD crystal structure (Swiss-Prot accession number for mouse MyoD: P10085, Protein Data Bank Id: 1MDY).

glutamic acid (mutation K145E) would prevent hydrogen bond formation between the phosphate group and the lateral chain, thus providing an explanation for the critical role of lysine in DNA binding. Similarly, a K145N missense mutation [28] is also likely to hamper DNA binding.

Remarkably, serine 144, the last residue of the loop in TWIST also appears to be involved in bHLH–DNA interaction as mutation to arginine eliminates TWIST binding capacities. Conservation of this amino acid among bHLH proteins is less remarkable than lysine 145, although it is present in more than 30% of hitherto identified bHLH transcription factors [29]. We suggest therefore, that conversion of serine into arginine may induce steric hindrance due to the bulky lateral chain of arginine and impair anchoring of the loop–helix II junction to DNA. Besides, persistence of DNA binding of the P139ins7 mutant despite loop elongation further indicates that the size of the loop plays a less critical role than the loop–helix II junction for protein–DNA interaction. The mechanism by which the P139ins7 genotype is converted into SCS phenotype remains unclear although reduced heter-

odimerisation with E12 and partial mis-localisation of the protein are likely to be involved.

Three other missense mutations in the basic domain of H-TWIST have been reported so far in SCS [17], two of which alter highly conserved residues involved in the formation of hydrogen bonds between phosphates and amino acid lateral chains (R116W and R120P). Based on the MyoD model, we would predict that they hamper binding to the *Nde*I E-box in a manner similar to the R118C mutation reported here. By contrast, the third mutation (Q119P) substitutes a residue which is conserved among Twist proteins across different species but to a lesser extent among other bHLH proteins (19% according to [29]), suggesting that glutamine might be involved in the functional specificity of the Twist basic domain.

The high degree of conservation between Twist bHLH motif and its target DNA sequence across species suggests, but does not prove, common target genes. Analysis in invertebrates suggests a direct link between Twist and genes encoding fibroblast growth factor receptors genes (*Fgfr*). This hypothesis is based on (i) the physical interaction of Ce-Twist with the promoter of *egl-15* (an *Fgfr* homologue) in *C. elegans* [9] and, (ii) the requirement of D-Twist for expression of *DFR-1* (one of the two *Fgfr* homologues) in *Drosophila* [30]. Considering that mutations in *H-TWIST* and *FGFR1-3* genes account for phenotypically related human craniosynostoses [31] and that pattern of *Fgfr* gene expression is altered in the sutures of heterozygous *M-Twist*-null mice [32], one can hypothesise that a direct relationship also exists between Fgfrs and Twist in vertebrates [32,33]. This remains as a speculation, however, since we could not detect any *Nde*I E-box in a 3-kb sequence spanning human *FGFR2* and *FGFR3* promoter regions (unpublished observations).

Heterozygous deletions of the *H-TWIST* gene are the most direct evidence for haploinsufficiency in SCS [19]. Likewise, defective transcriptional regulation of *H-TWIST* through a positional effect has been evoked as another mechanism that could account for the disease [34]. From our results (this study and [21]), we conclude that mutations in the *H-TWIST* gene can cause skeletal abnormalities by three other mechanisms, all of which result in a loss of function of the TWIST protein. These are: (i) protein instability, (ii) loss of dimerisation leading to abnormal cellular localisation and (iii) loss of DNA binding activity. SCS thus represents an original genetic model for analysing the critical functions of different subdomains in bHLH proteins.

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