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Mechanism of Transcriptional Activation by the Proto-oncogene Twist1*◆

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Mammalian Twist1, a master regulator in development and a key factor in tumorigenesis, is known to repress transcription by several mechanisms and is therefore considered to mediate its function mainly through inhibition. A role of Twist1 as transactivator has also been reported but, so far, without providing a mechanism for such an activity. Here we show that heterodimeric complexes of Twist1 and E12 mediate E-box-dependent transcriptional activation. We identify a novel Twist1 transactivation domain that coactivates together with the less potent E12 transactivation domain. We found three specific residues in the highly conserved WR domain to be essential for the transactivating function of murine Twist1 and suggest an α-helical structure of the transactivation domain.

When the human genome was sequenced, one of the surprising results was the low number of genes, summing up to only about one-tenth of the expected number. Explanations for this apparent lack of genes were found in differential gene splicing and protein modification as well as in the combinatorial use of regulating signals and transcription factors. A third explanation, multifunctional proteins, has so far received little attention. Here we show that the basic helix-loop-helix (bHLH)[2] protein Twist1, a known transcriptional inhibitor, can also function as an activator, depending on the regulatory sequences of the target genes. Identification of this additional molecular mechanism may aid in explaining the pleiotropic effects of Twist1 in development and in understanding the complex phenotype of the human Saethre-Chotzen syndrome, which is caused by haploid insufficiency of Twist1.

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[2] The abbreviations used are: bHLH, basic helix-loop-helix; AD, activation domain; nls, nuclear localization signal; Luc, firefly luciferase; EGFP, enhanced green fluorescent protein; CDS, coding sequence; EMSA, electrophoretic mobility shift assay; NT, amino-terminal; CT, C-terminal; D-Twist, *Drosophila* Twist; FGFR, fibroblast growth factor receptor; MSV, murine sarcoma virus; WT, wild type; CBP, CREB-binding protein; CREB, cAMP-response element-binding protein; PCAF, p300/CBP-associated factor.

Tissue-specifically expressed bHLH transcription factors are important regulators during embryonic development and postnatal life. They mediate their function through binding to DNA elements of the NCANNTGN consensus sequence termed E-boxes (1, 2). The evolutionarily conserved molecular mechanisms leading to DNA binding have been firmly established. In brief, two amphipathic α-helices connected by a loop region form the HLH motif, a protein interaction domain through which bHLH factors form homo- or heterodimers. A region of basic residues N-terminal to the HLH motif is necessary for DNA binding. Members of a class of ubiquitously expressed bHLH factors termed E-proteins serve as activating dimerization partners for tissue-specific bHLH factors (3, 4). In general, single E-boxes are sufficient for bHLH responsiveness, yet cooperative binding to dual E-boxes has been observed (5). Id proteins constitute a specific class of inhibitory HLH factors, which are unable to bind DNA due to a lack of basic regions (6). The Id proteins consequently function as negative regulators.

Although dimerization and DNA binding are mechanistically similar for all bHLH proteins, the transactivational mechanisms are often of different evolutionary origin. The closely related myogenic bHLH factors Myf5 and MyoD1 both up-regulate muscle-specific genes such as muscle creatine kinase, yet the sequences of their activation domains (AD) are unrelated (7, 8).

DNA binding by bHLH factors is not necessarily followed by transactivation. E-proteins, which transactivate immunoglobulin genes (9) and Twist1, are both capable of binding to muscle creatine kinase E-boxes as either homodimers or heterodimers yet without being able to induce transcription (10, 11). The internal and the flanking bases of the E-box may determine the functional response as even very simple reporter constructs reveal the gene specificity of bHLH complexes. Other cellular factors such as kinases and acetyltransferases can also regulate the function of bHLH factors by modifying dimerization, DNA binding, or even the activity of transactivation domains (12–15).

The bHLH factor Twist was first identified as a mesodermal specifier in *Drosophila* (16–18), where it, potentially in synergy with Dorsal, activates genes such as *Tinman*, *D-Mef2*, *Rhomboid*, *Snail*, and *Heartless* (19–22). *D-Mef2* and most likely also *Tinman* are induced by direct binding of *Drosophila* Twist (D-Twist) homodimers to enhancer E-boxes (23–26). D-Twist thus induces cellular differentiation by direct transcriptional activation.

It was therefore surprising that the mouse homologs Twist1 and Twist2 did not specify cellular differentiation but

actually inhibited myogenic differentiation (27, 28). Further studies revealed that Twist proteins directly inhibit a number of myogenic transactivators including the bHLH factors MyoD1 and Myf5, the MADS transcription factor Mef2, and the acetylating co-activators p300/CBP and PCAF (10, 11, 29, 30). The mammalian Twist proteins thus utilize several mechanisms to inhibit transcriptional activation of myogenic targets. The mammalian Twist proteins also directly inhibit the transactivation domain of the Dorsal homolog NFκB (31), thereby providing a negative feedback, which contrasts the D-Twist/Dorsal synergy observed in *Drosophila*.

Twist1 also functions as a proto-oncogene by protecting cells from p53-dependent apoptosis (32). The direct inhibition of the p53 transactivation domain by Twist1 and E1A (30) suggests inhibition of the p300/PCAF co-activators. These co-activators are also involved in myogenic transactivation (33, 34), where they indeed are directly inhibited by Twist1 (30). In addition to its anti-apoptotic function, Twist1 plays a role in the epithelial-mesenchymal transition (EMT). In epithelial-derived tumors, overexpression of Twist1 results in epithelial-mesenchymal transition due to down-regulation of epithelial markers such as E-cadherin and up-regulation of mesenchymal markers such as N-cadherin (35), which is directly up-regulated by Twist1 (36). Also, other tumor-related genes such as *akt2* and *gli1* have been suggested to be targets of Twist1 mediated up-regulation (37, 38). Overexpression of Twist1 therefore promotes development of malignant tumors by supplementing the anti-apoptotic role with an increased rate of metastasis. Consistently, Twist1 has been shown to be overexpressed in several types of cancer (32, 35, 39–43). Interestingly, the function of Twist1 as an inducer of epithelial-mesenchymal transition may reflect a role in embryonic development (44, 45), particularly during the migration of neural crest cells (46, 47).

In humans and mice, the haploid insufficiency of Twist1 results in a variety of developmental defects described in humans as Saethre-Chotzen syndrome (48–50). The Saethre-Chotzen syndrome frequently includes cranial malformations, which suggests an involvement of Twist1 in the cranial formation. Furthermore, Twist1 and Twist2 both inhibit osteogenic differentiation (51, 52) by direct inhibition of the osteogenic transcription factor Runx2 (53), of which transcription is also induced by Twist1 (54). In summary, the majority of activities assigned to the mammalian Twist proteins indicates inhibitory functions in a number of different processes.

In contrast to these antagonizing functions of Twist1, a positive correlation has in several contexts been found between the expression of Twist1 and the osteogenic factor FGFR2 (55–57). Consequently, the mammalian Twist proteins most likely function as inducers in the early phases and inhibitors in later differentiation, thereby playing a dual role in osteogenesis. A direct transactivation of mammalian FGFRs would parallel observations from invertebrates, where positive targets of Twist in *Drosophila* include the FGFR *Heartless* (21), and in *Caenorhabditis elegans*, the FGFR *egl-15* (58). In *Drosophila*, the transactivation appears to be mediated by homodimers of D-Twist (26), whereas in *C. elegans*, heterodimers of CeTwist and E-proteins (Daughterless) most likely mediate the transactivation (58, 59). In mammals, homodimers of Twist1 up-regulate transcription of FGFR2 and periostin, whereas transcription of thrombospondin is induced by heterodimers of Twist1 and E12 (57, 60). The choice of E-box target sequences may thus be determined by the dimerization partner. When *Twist* genes from *C. elegans, Drosophila*, and human were expressed in *Drosophila* imaginal wing discs, distinct functional differences were observed (61). Although the different Twist proteins have functionally diverged, properties such as the positive regulation of FGFRs are apparently conserved. It should, however, be noted that transactivation of proposed Twist1 target genes is not necessarily direct. Twist1 could inhibit the induction of a repressor and thereby increase transcription of "indirect target genes." Such an indirect up-regulation by Twist1 has been reported (27), and a direct assay is needed to rule out this possibility.

Here we report a mechanism of direct transcriptional activation by murine Twist1. We provide evidence that in mammalian cells, Twist1-E12 complexes bind E-box elements and thereby activate transcription. We further identify a transactivation domain in the C terminus of Twist1, which functions independently of and more potently than the E12 activation domain AD2. Finally, we have identified three specific residues essential for the transactivating function of murine Twist1. The spacing of these residues suggests that the activation domain adopts an α-helical structure.

EXPERIMENTAL PROCEDURES

Plasmids

Gene-specific Reporters—The pCaSpeR-*Tinman*::*lacZ* (pTinE1/E2/E3, courtesy of Dr. M. Frasch) was used as a Twist-responsive reporter (24). From this reporter, which contains three *Tinman* E-boxes, constructs pTinE1/E2, pTinE3, and placZ were made, which contained two, one, and no *Tinman* E-boxes, respectively (see Fig. 1*B* for details). The pCaSpeR-*Mef2*::*lacZ* (courtesy Dr. R. Cripps) was used as an alternative Twist-responsive reporter (25). The *pMyf4*::*CAT* (pMyf4, courtesy Dr. H. H. Arnold) was used as a myogenic reporter (62).

Expression Vectors—The pEMSV scribe α2 (pEMSV (63)) plasmid served as expression vector for all transfactors used in this study. Expression vectors for Twist1 deletion constructs were constructed by PCR-directed strategies on pEMSV Twist (10) followed by conventional cloning. Residues of the murine Twist1 (GenBank^{TM} accession number AAH83139) expressed by these constructs include amino acids 2–206 (nlsTw), 113–206 (nlsTwΔNT), 2–166 (nlsTwΔCT), 2–111 (nlsTwNT), 113–169 (nlsTwbHLH), and 170–206 (nlsTwCT), respectively. Each truncated mutant was preceded by a start codon followed by an SV40 nuclear localization signal (MDPKKKRKLRSTP-, nls). As a positive control for activation, D-Twist was used. For comparing expression levels, the D-Twist CDS was cloned from pCS2 D-Twist (courtesy Dr. A. Firulli) into the EcoRI site of pEMSV. For expression of a dimerization partner for the murine Twist1, the previously described pEMSV E12 (2) was used. A full-length E12 CDS was cloned from pcDNA3 $E12_{WT}$ (courtesy Dr. R. Benezra) into the EcoRI site of pEMSV. In addition, an expression vector was constructed by conventional

cloning for expression of an E12 mutant devoid of the entire N-terminal region. Residues of the human E12 factor (E2A, GenBank accession number AAA61146) expressed by these three constructs include amino acids 217–654 (E12), 1–654 ($E12_{WT}$), and 415–654 (E12ΔNT), respectively. Constructs for expression of Twist1 and E12 factors tethered by a flexible $(GGGS)_5$-polylinker were made by conventional cloning, linking the following polypeptides: $1–206_{Twist1}$~$2–206_{Twist1}$ (Tw~Tw), $1–206_{Twist1}$~$227–654_{E12}$ (Tw~E12), $217–654_{E12}$~$2–206_{Twist1}$ (E12~Tw), and $217–654_{E12}$~$227–654_{E12}$ (E12~E12), respectively. Specific point mutations were introduced into Twist1 and E12 expression vectors using QuikChange mutagenesis kit (Stratagene) on pEMSV templates. The coding region of each construct was verified by sequencing to contain only the desired point mutation (BigDye 3, Applied Biosystems).

The pEMSV Myf5 construct (64), which expresses human Myf5 (GenBank accession number AAH69373), served as positive control for transactivation of the myogenic reporter. In addition, constructs were made for expression of a C-terminally truncated Myf5 (residues 1–126, Myf5ΔCT) and a Myf5ΔCT-TwCT fusion factor (residues $1–134_{Myf5}$~$165–206_{Twist1}$). The pEMSV Myf5ΔCT and pEMSV Myf5-TwCT were constructed by PCR-directed strategies on pEMSV Myf5. An expression vector for firefly luciferase (Luc) was constructed by conventional cloning of the Luc CDS from pGL3-Control (Promega) into pEMSV. Similarly, an expression vector for enhanced green fluorescent protein (EGFP) was constructed by conventional cloning of the EGFP CDS from pEGFP-C1 (Clontech) into pEMSV. The two vectors were used as quantitative (Luc) and visual (EGFP) controls, respectively.

Cell Culture

The HT1080 human cell line (65) and the C_2C_{12} murine myoblast cell line (66) were each propagated in growth medium consisting of Dulbecco's modified Eagle's medium (Invitrogen) supplemented with fetal calf serum (10%) and sodium pyruvate (1%). One day prior to transfection, cells were trypsinized and distributed in a density that, upon overnight incubation, would yield cells at ~50% confluence. The transfections were performed using a mix of 1.0 μg of reporter plasmid, 1.0 μg of E12 (or empty) expression vector, 1.0 μg of Twist1 (or empty) expression vector, and 0.25 μg each of the Luc and EGFP expression vectors. Cells evenly distributed in triplicate 35-mm wells were accordingly transfected with a total of 3.5 μg of DNA per triplicate. HT1080 cells were transfected using SuperFect reagent (Qiagen) according to the manufacturer's instructions. After transfection, the medium was changed to growth medium. After a 24-h incubation, the cells were collected and harvested. C_2C_{12} cells were transfected using Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. After overnight incubation in growth conditions, the medium was changed to differentiation conditions (2% fetal calf serum). The cells were collected 48 h later and harvested.

Reporter Assays

Confluent cells were harvested in 50 mM Tris-HCl, pH 7.2, 1.0 M EDTA, 150 mM NaCl, resuspended in 50 μl of Tris-HCl (0.25 M, pH 8.0) per 35-mm dish, and lysed by three freeze/thaw cycles. After centrifugation, the soluble supernatant was used for analysis. For quantification of lacZ reporter activity, β-galactosidase assays were performed on 5–10 μl of cellular extract using the Galacton system (Applied Biosystems) (67). Firefly luciferase assays were performed on 5–10 μl of cellular extract using 50 μl of 10% luciferase assay reagent (Promega) in Tris-HCl (0.25 M, pH 8.0) and measured immediately with a luminometer. Chloramphenicol acetyl transferase assays were performed as described previously (68). Quantification of Luc expressed from a co-transfected MSV-driven reporter plasmid demonstrated that transfection efficiencies differed insignificantly within a single setup. Also, transfection efficiencies both within single setups and between independent setups were estimated visually (EGFP) to be highly similar. Within single setups, the activity of the gene-specific reporter was therefore used directly as a measure for transcriptional activation, whereas independent assays were correlated by setting the average Twist1-E12 transactivation to 100% (each assay included 4–6 measurements; five independent assays thus represent 20–30 measurements).

Protein Procedures

The E-box E3 sequence of *Tinman* was used to design a double-stranded oligonucleotide (ccccccCAACATATGGCG-cccccc, *Tinman* sequence in capitals, E-box underlined), which served as probe for electrophoretic mobility shift assays (EMSA). Single-stranded oligonucleotides were labeled by kinase reaction using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs) and then mixed, denatured, and annealed. Unincorporated nucleotides were removed on a spin column (Qiagen nucleotide removal Kit). EMSAs were performed in 20-μl binding reactions as described previously (4). For supershift assays, 0.5 μl of specific antibody was added. For competition assays, 5.0 pmol of unlabeled double-stranded probe with either a WT or a mutated (ccccccCAATCT-GAAGCGcccccc, mutated bases underlined) E-box was added. Western blot assays were performed according to standard protocols using E12-CT (V-14: sc349)-, Twist1-CT (C-17: sc6269)-, or Twist1-NT (N-19: sc6070)-specific primary antibodies in recommended dilutions (Santa Cruz Biotechnology). Horseradish peroxidase-coupled antibodies (Dako) were used as secondary antibodies, anti-rabbit for V-14 and anti-goat for C17/N19, respectively. Chemiluminescence detection reactions were performed using ECL detection reagent (Amersham Biosciences).

RESULTS

Murine Twist1 Transactivates the Tinman Reporter in an E-Protein-dependent Manner

To establish the mechanisms of Twist1 transactivation, we utilized the *Tinman*-derived minimal reporter construct (pTinE1/E2/E3), which in *Drosophila* is known to be D-Twist-responsive (24). By employing the reporter in a mammalian context, we simulated conditions in which Twist1 transactivation may naturally occur.

Expression of either Twist1 or E12 alone resulted in no significant activation of the reporter. In contrast, coexpression of Twist1 and E12 resulted in a more than 100-fold increase in

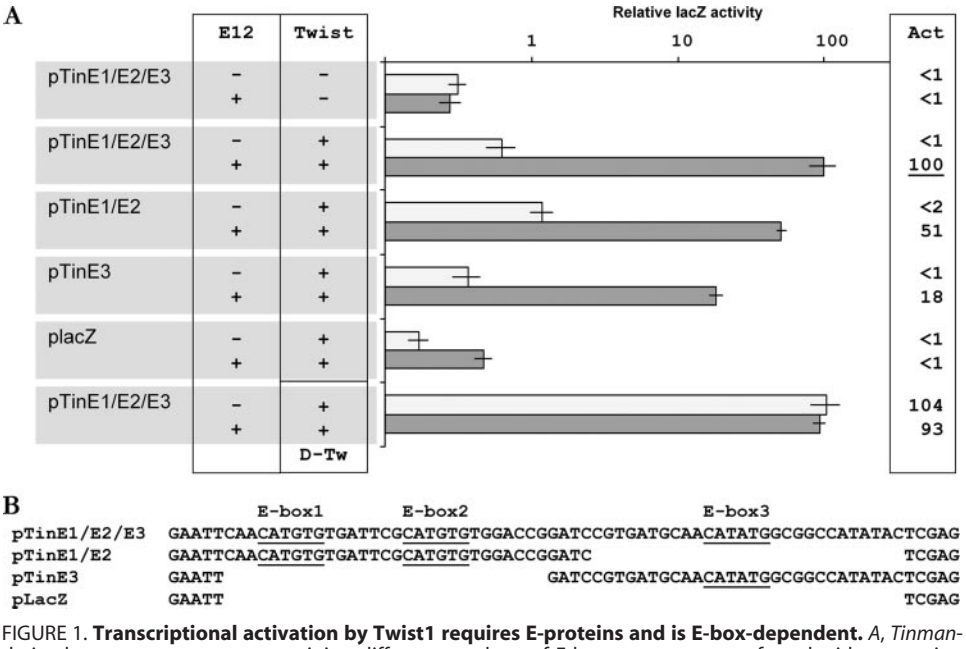

FIGURE 1. **Transcriptional activation by Twist1 requires E-proteins and is E-box-dependent.** *A*, *Tinman*-derived reporter constructs containing different numbers of E-boxes were co-transfected with expression vectors for E12, Twist1, D-Twist (*D-Tw*), or combinations as indicated. The resulting reporter activation (Act) is given in arbitrary units. Activity of pTinE1/E2/E3 achieved by coexpression of Twist1 and E12 was set to 100%; note the logarithmic scale. The data are from a single representative experiment. *B*, schematic representation showing the complete sequences of the different E-box regions of the reporters (E-boxes are *underlined*).

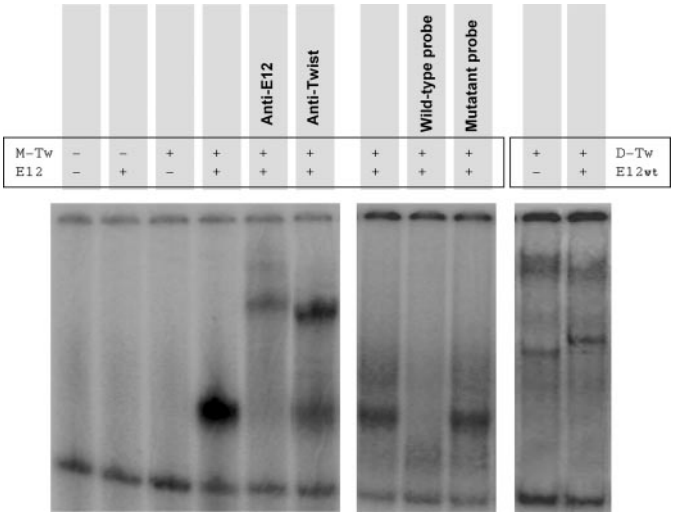

FIGURE 2. **Heteromeric Twist1-E12 complexes bind specifically to *Tinman* E-boxes.** Cellular extracts from transfected cells were assayed for the ability to specifically retard migration of an E-box E3-containing probe. A specific band appeared only when Twist1 (*M-Tw*) and *E12 were coexpressed. This band was supershifted by antibodies specific for either E12 or Twist1. The addition of an excess of unlabeled wild-type probe specifically ablated the band, whereas unlabeled mutant probe had no effect. In contrast to Twist1, D-Twist (D-Tw*) bound the probe both as a homodimer and as a heterodimer.

reporter activity (Fig. 1*A*). Similar results were obtained with Twist2 (Dermo1, not shown). Expression of D-Twist alone resulted in a potent reporter activity, which was unchanged by coexpression of E12 (or $E12_{WT}$, not shown) (Fig. 1*A*). Use of a *D-Mef2*-derived reporter gave similar results (not shown).

In *Drosophila*, activation of the *Tinman* reporter is E-box-dependent (24). To verify that this is true also for Twist1, we tested the role of E-boxes for transactivation. Deletion of E-box E3 (pTinE1/E2) reduced the transactivation to 50%. Similarly, deletion of E-boxes 1 and 2 (pTinE3) reduced the transactivation to 20%. Deletion of all three E-boxes (placZ) reduced transactivation to less than 1% when compared with the original pTinE1/E2/E3 reporter. As for pTinE1/E2/E3, the expression of Twist1 alone (or E12 alone, not shown) resulted in reporter activities similar to background (Fig. 1*A*). Twist1 transactivation of the *Tinman* reporter is thus E-box-dependent and requires the presence of E-proteins.

The fact that both pTinE1/E2 and pTinE3 are transactivated only by coexpression of Twist1 and E12 suggests that there are no functional differences between E-boxes E1/E2 and E3. A sequence comparison of the E-boxes points to NCATNTGT (E-box underlined) as a potential target consensus (Fig. 1*B*, E-box E3 in reverse orientation). Importantly, this consensus also matches the conserved E-box (E1) of the *D-Mef2* reporter (25).

To verify direct binding of Twist1-E12 complexes to the E-boxes of the reporter, we performed an EMSA. A probe containing the *Tinman* E-box E3, which was sufficient for reporter activation (Fig. 1*A*), was assayed for retardation by cellular extracts from cells expressing Twist1, E12, or both. Only extract from cells coexpressing Twist1 and E12 produced a specific band, which furthermore was supershifted by the addition of either E12-specific or Twist-specific antibodies (Fig. 2). The DNA binding was sequence-specific because the addition of wild-type probe completely abolished the band, whereas the addition of mutant probe had no effect (Fig. 2). Comparable results were obtained using E-boxes E1 and E2 (not shown). These results demonstrate that murine Twist1 can bind to a *Tinman*-specific E-box sequence but requires heteromerization with E-proteins. In contrast, D-Twist bound to the *Tinman* E-box E3 as homodimers in the absence of E-proteins but as heterodimers when coexpressed with E12 (Fig. 2). Due to the similar sizes of D-Twist and E12, the larger full-length E12 ($E12_{WT}$) was used to demonstrate formation of heterodimers. The differential dependence of Twist1 and D-Twist on E-proteins for transactivation (Fig. 1*A*) is thus also reflected in their ability to bind to the *Tinman* E-box E3.

Transcriptional Activation by Tethered Factors

To assess the Twist1:E12 stoichiometry of the transactivating complex in cells, a series of constructs was made, each of which expressed two bHLH factors joined in a tail-to-head fashion by a polypeptide linker. The tethering has two important implications. First, the joined factors are co-translated and therefore present in cells in an exact 1:1 ratio. Second, the tethering is expected to favor interaction between the joined factors

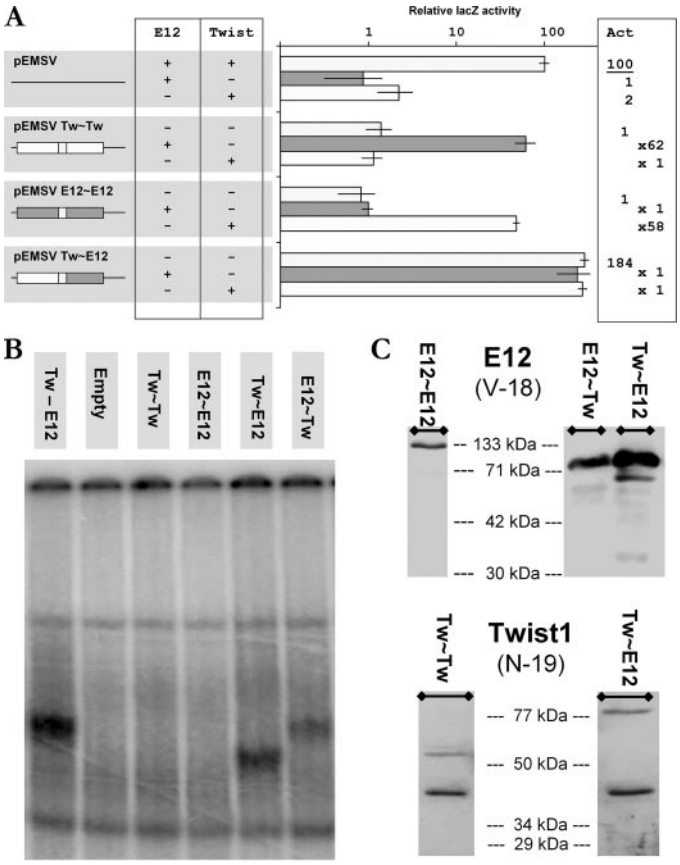

FIGURE 3. **Tethered hetero- but not homodimers mediate transcriptional activation.** *A*, reporter activation (*Act*) by tethered dimers expressed either alone or with monomers of E12 or Twist1 as indicated. The tethered homodimers Tw~Tw and E12~E12 were only active when coexpressed with the complementary monomer. In contrast, the tethered heterodimers of either orientation activated the reporter independently of monomer expression (only shown for Tw~E12). Reporter activation is given in arbitrary units. Activity achieved by coexpression of Twist1 and E12 was set to 100% (note the logarithmic scale). The data are based on two independent experiments. *B*, cellular extracts from transfected cells were assayed for the ability to specifically retard migration of an E-box E3 containing probe. Only the tethered heterodimers but not the homodimers were able to bind to E-box E3. See "Discussion" for consideration of the different retardations. *C*, protein sizes of the tethered dimers were verified by denaturing SDS-PAGE of cellular extracts followed by Western blotting. The tethered factors were detected using specific antibodies directed against either Twist1 or E12. The observed bands correspond to the expected sizes (Tw~Tw, 44 kDa; E12~E12, 90 kDa; Tw~E12 and E12~Tw, 67 kDa).

(69), which thereby are prevented from interacting with cellular bHLH factors. Twist1 and E12 factors tethered in all four possible dimeric conformations were each assayed for their transactivating abilities on the *Tinman* reporter.

Both tethered homodimers Tw~Tw and E12~E12 failed to activate the *Tinman* reporter and thus behaved no differently from the respective monomers described above. In contrast, expression of tethered heterodimers Tw~E12 (or E12~Tw, not shown) resulted in transcriptional activation of the reporter similar to that achieved by coexpression of Twist1 and E12 monomers (Fig. 3*A*). These results confirm that reporter transactivation requires both Twist1 and E12.

Tethered Homodimers Are Capable of Interacting with Monomers—Based on previous reports, the tethered homodimers are expected to form intramolecular dimers that are resistant to monomer titration (57, 69). To determine whether this is indeed the case, Tw~Tw and E12~E12 were coexpressed with the complementary monomer, *i.e.* E12 and Twist1, respectively. Each tethered homodimer was also coexpressed with its monomeric form to exclude trivial quantitative effects.

Upon coexpression of tethered Twist1 homodimers with E12 monomers or coexpression of E12 homodimers with Twist1 monomers, the reporter was potently activated (Fig. 3*A*). The same was observed for coexpression of Tw~Tw and E12~E12 homodimers (not shown). Conversely, the coexpression of Twist1 homodimers with Twist1 monomers or of E12 homodimers with E12 monomers had no transactivating effect (Fig. 3*A*). The tethered homodimers are under these conditions concluded to be able to interact with monomers.

Equimolar Amounts of Twist1 and E12 Are Present in the Transactivating Complex—In the tethered heterodimers, Twist1 and E12 are expressed in an exact 1:1 ratio. Should, however, the active complex be composed of a different ratio of Twist1 and E12 (*e.g.* 1:3 or 3:1), then one of the partners would be underrepresented in cells expressing the tethered constructs, and an increase of this factor should lead to an elevated activation of the reporter. To test this possibility, we coexpressed Twist1 and E12 monomers with the tethered heterodimers.

The transactivation achieved by Tw~E12 or by E12~Tw was not significantly affected by coexpression either of Twist1 monomers (0.9- and 2.1-fold, respectively) or of E12 monomers (0.8- and 2.4-fold, respectively) (Fig. 3*A* and not shown). Thus, in cellular conditions, the complex transactivating the *Tinman* reporter is evidently composed of Twist1 and E12 in a 1:1 ratio. Similar results were obtained by quantitatively titrating Twist1 and E12 monomers in coexpression experiments (not shown).

The Tethered Heterodimers Form Intramolecular DNA-binding Complexes—The transcriptional activation by the heteromeric tethered monomers is expected to be mediated by intramolecular dimers (unimolecular) but could also depend on the formation of intermolecular tetramers (bimolecular). To distinguish between these two possibilities, we performed EMSAs using extracts from transfected cells. Although neither of the homodimers produced any specific band retardation, each of the tethered heterodimers displayed specific E-box binding. The E12~Tw band migrated with a mobility similar to that observed for coexpressed Twist1 and E12 monomers, which supports the model of intramolecular dimerization. In contrast, the Tw~E12 band migrated even faster, probably due to a more compact conformation (Fig. 3*B*, see "Discussion" for details). Since similar results were obtained with *in vitro* translated proteins (not shown), the increased migration is unlikely to be caused by post-translational modifications.

As coexpression with the complementary monomer functionally changed the tethered homodimers into transactivators, we presume that one or two monomers "break open" the tethered homodimers, forming tri- or tetrameric complexes. We observed such higher order bands in the EMSA but only when using a probe containing two E-boxes (not shown).

To validate the actual sizes of the tethered dimers, we performed Western blots under denaturing conditions using E12-

and Twist1-specific antibodies. With an E12-specific antibody, a band of the expected 90 kDa was detected for the tethered E12 homodimer, whereas bands matching the expected 67 kDa were detected for both Tw∼E12 and E12∼Tw. Using a Twist-specific antibody, we detected a band of the expected 44 kDa for the tethered Twist homodimer and a band of the expected 67 kDa for the Tw∼E12 tethered heterodimer (Fig. 3*C*). The Western blots thus verify expression of correctly sized proteins, thereby indicating that the increased migration of Tw∼E12 is caused not by a truncation but, most likely, by steric restraints, which are not present in the native Twist1-E12 dimer or in the E12∼Tw tethered heterodimer.

In summary, the tethered homodimers appeared in our experiments to be functionally identical to monomers but with an increased tendency to form tetrameric complexes with the complementary monomers. In contrast, the tethered heterodimers formed intramolecular heterodimers, which were functionally identical to Twist1 and E12 when coexpressed in equimolar ratios.

Twist1 Transactivates Even in the Absence of an E12 Activation Domain

Transcriptional activation of the *Tinman* reporter depends on both Twist1 and E12. As E12 and $E12_{WT}$ contain one and two transactivation domains, respectively (70–72), it is possible that Twist1 contributes only indirectly to the reporter activation, *e.g.* by providing DNA sequence specificity. To test this possibility, we deleted the ADs of E12 so that any remaining activity could be attributed to Twist1. $E12_{WT}$ is subject to complex functional regulations (73), and in studies of bHLH factors, a truncated version lacking AD1 (E12) is frequently used as a heterodimerization partner. When coexpressed with Twist1, $E12_{WT}$ and E12 resulted in similar levels of reporter transactivation (92 and 100%, respectively). The E12 thus behaves similarly to $E12_{WT}$, which suggests that the E12-AD1 does not significantly contribute to the reporter activation. To assess the functional role of AD2 in the Twist1-E12 complex, we constructed two mutant versions of E12 in which the AD2 was either deleted (E12ΔNT) or perturbed by point mutations ($E12_{mut}$AD2, VL403ER), which was previously shown to abolish the function of AD2 (70). When Twist1 was coexpressed with either E12ΔNT or $E12_{mut}$AD2, similar low levels of reporter transactivation were achieved (35 and 47%, respectively) (Fig. 4*A*). Neither of the E12 proteins mediated any transactivation in the absence of Twist1 (not shown). Thus, Twist1-E12 transactivation relies in part on the transactivating properties of E12-AD2 but not on those of E12-AD1. The fact that Twist1-E12 dimers void of all known E12 ADs still significantly transactivate the *Tinman* reporter shows that Twist1 directly contributes to the Twist1-E12 transactivation.

To verify that the reduced transactivation by the NT-truncated mutants was not simply caused by perturbing either dimerization or DNA binding of the complex, we tested $E12_{WT}$ and the two E12 deletion-mutants together with Twist1 in an EMSA. The E12 mutants each displayed band retardation corresponding to its respective size when dimerized to Twist1 (Fig. 4*B*). Both mutant proteins and $E12_{WT}$ are thus produced and capable of DNA binding.

The CT Region of Twist1 Is Required for Transactivation

To identify the Twist1 domain required for transactivation, we created a series of Twist1 mutations. The mutants were each fused to an SV40 nls to ensure nuclear localization even in cases where our mutagenesis might have impaired an nls in the Twist1 protein. The Twist1 mutant constructs included the following domains of Twist1: Full-length (nlsTw), N-terminally truncated (nlsTwΔNT), C-terminally truncated (nlsTwΔCT), N-terminal region (nlsTwNT), bHLH domain (nlsTwbHLH), or C-terminal region (nlsTwCT).

When E12 was coexpressed with either nlsTw or nlsTwΔNT, comparable levels of reporter transactivation were achieved (100 and 102%, respectively), and no difference was observed between full-length Twist1 with or without nls (not shown). In contrast, when E12 was coexpressed with either nlsTwΔCT or nlsTwbHLH, only very low levels of reporter transactivation were achieved (7 and 12%, respectively). Coexpression of E12 with either nlsTwNT or nlsTwCT, both lacking the bHLH domain, as expected, did not transactivate the reporter (Fig. 5*A*). Neither of the Twist1 mutant proteins mediated any transactivation in the absence of E12 (not shown). In summary, the Twist1 bHLH domain is required for Twist1-E12 transactivation, yet the transactivation is potently increased if the Twist1-CT is preserved. In contrast, transactivation occurred independent of the Twist1-NT region.

To verify that the reduced activity of the CT-truncated mutants was not simply caused by perturbing their dimerization with E12 or their DNA binding capacity, we assayed the DNA binding properties of the four transactivating Twist1 mutant proteins, each of which displayed band retardation corresponding to its respective size when coexpressed with E12 (Fig. 5*B*). All mutant proteins are thus expressed and capable of DNA binding. Protein misfolding or instability can therefore not explain the loss of transactivational activity associated with the loss of the Twist1 CT. This strongly indicates that a transactivation domain is located C-terminally to the bHLH domain of Twist1. The Twist1-CT thus contributes significantly to the Twist1-E12 transactivation, whereas the Twist1-NT has no direct effect.

The Twist1-AD Is More Potent than the E12-AD2

The individual mutagenesis assays of Twist1 and E12 suggest that the Twist-AD is the major contributor to the Twist1-E12 transactivation. This quantitative comparison is indirect, and to compare directly the contribution of each activation domain, we tested various combinations of Twist1- and E12 mutant proteins in the same assay.

Deleting or mutating the E12-AD2 reduced the transactivation to ∼50%, independent of whether the dimerization partner was full-length Twist1 (from 100 to 50%) or a CT truncation of Twist1 (from 20 to 10%). In contrast, deletion of the Twist1 CT resulted in an 80% reduction of reporter activation, also independent of whether the dimerization partner was E12 (from 100 to 20%) or an AD2 mutation of E12 (from 50 to 10%) (Table 1).

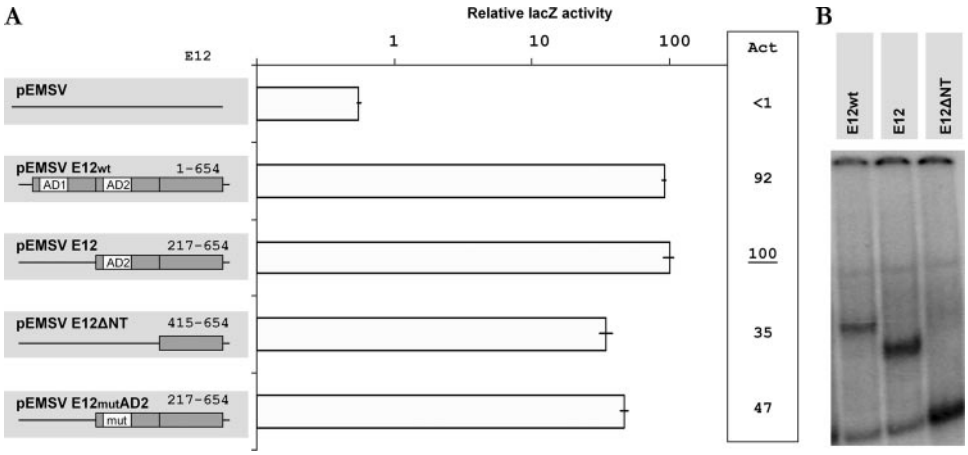

FIGURE 4. **Twist1 transactivates even in absence of an E12 activation domain.** *A*, reporter activation (*Act*) after coexpression of Twist1 with E12 wild-type or mutant constructs. Loss of E12-AD1 has no effect, whereas loss of E12-AD2 reduces transactivation. When both E12-ADs are lost, the remaining 35–47% activity must be attributed to Twist1. Reporter activation is given in arbitrary units. Activity achieved by coexpression of Twist1 and E12 was set to 100% (note the logarithmic scale). The data are from a single representative experiment. *B*, EMSA demonstrated that DNA binding of the Twist1-E12 complex to E-box E3 was not hampered by the deletions. Neither transcriptional activation nor DNA binding was observed in the absence of Twist1 (not shown).

The two activation domains can thus be considered to be functionally independent. Furthermore, the activity with both ADs intact is 10-fold the activity when compared with the activity when both ADs are deleted. To this 10-fold increase, the Twist1-AD contributes 5-fold, whereas the E12-AD2 contributes only 2-fold. The Twist1-AD is thus more potent in transactivating the reporter than the E12-AD2.

The Twist1 Residues 165–206 Are Sufficient for Transactivation

We next asked whether the transactivation by the Twist1-AD is a property that can be transferred to a different transcriptional context. To test this possibility, we exchanged the CT of Myf5 (residues 136–255), which is necessary for activation of the muscle-specific reporter *pMyf4*::*CAT* (8), with the CT of Twist1 (residues 165–206), and assayed the chimeric construct for restored transactivation. It should be noted that transactivation of this reporter by myogenic bHLH factors is potently inhibited by Twist1. Expression of E12 or Twist1 alone or together does not increase transcription of the reporter (10).

Together with E12, Myf5 potently activated the *Myf4*-reporter, whereas deletion of the Myf5-CT abolished this activity almost completely. Fusion of the Twist-CT to the Myf5ΔCT significantly restored the transactivation capacity (Fig. 6). Residues 165–206 of Twist1 thus constitute a domain sufficient for functional transactivation.

Three Residues of the Twist1 WR Domain Are Essential for Transactivation

To further map the transactivation domain of Twist1, a series of point mutations was created. Because a deletion assay had shown that residues 196–206 do not contribute to transactivation (not shown), the point mutations were distributed within residues 187–195. Furthermore, as deletion of the entire Twist1-AD still resulted in 20% transactivation (Table 1), the point mutants were expected to display activities between 20 and 100%. Each mutant was therefore rated based on its relative transactivation as being a weak (<50%), intermediate (50–75%), or strong (>75%) transactivator (summarized in Table 2). Although each of the point mutations displayed DNA binding similar to wild-type Twist1 in EMSA (not shown), the reporter transactivation was significantly reduced when the residues Leu-187, Phe-191, or Arg-195 were mutated (Table 2). Interestingly, these three amino acids are located in the highly conserved WR region (74) and are furthermore the only residues completely conserved throughout the animal kingdom (Fig. 7).

DISCUSSION

We demonstrate here direct transcriptional activation by the murine bHLH factor Twist1, which has so far mainly been associated with transcriptional inhibition. This transactivating function of Twist1 parallels the one described for D-Twist (26), yet the dimerizing requirements of the two factors differ considerably. When assayed under the exact same conditions, Twist1 required coexpression of E-proteins, whereas D-Twist was capable of potent transactivation both with and without E-proteins. This demonstrates a conserved function of Twist as transactivator yet also a divergence in the mechanism. The strict requirement for both Twist1 and E12 argues that under our experimental conditions, E-box binding and transactivation by Twist1 is mediated exclusively by heterodimers. *In vitro* DNA binding assays support this finding; Twist1 retarded migration of the E-box probe only when coexpressed with E12, whereas D-Twist was able to do so both as homodimer and when heterodimerized with E12. Furthermore, using Twist1- and E12-specific antibodies, we demonstrated the presence of both Twist1 and E12 in the complex, which binds to the E-box sequences of the reporter.

The *in vitro* DNA binding of Twist1 has, on several occasions and for different E-boxes, been shown to require the presence of E-proteins (10, 11, 75), yet transactivation of FGFR2 and periostin and binding of Twist1 to the muscle creatine kinase E-box has recently been reported also in the absence of E-proteins (57). It remains to be established whether these observations reflect differences in the specific experimental settings or in the physiological state of the Twist1 protein. However, under conditions where D-Twist homodimers bound DNA and activated transcription, neither of these functions was observed for Twist1.

Tissue-specific bHLH factors are *in vivo* expected to heterodimerize mainly with E-proteins, which are alternative splice products of a number of different genes (71, 76). The minute yet reproducible activation by Twist1 alone (Fig. 1*A*)

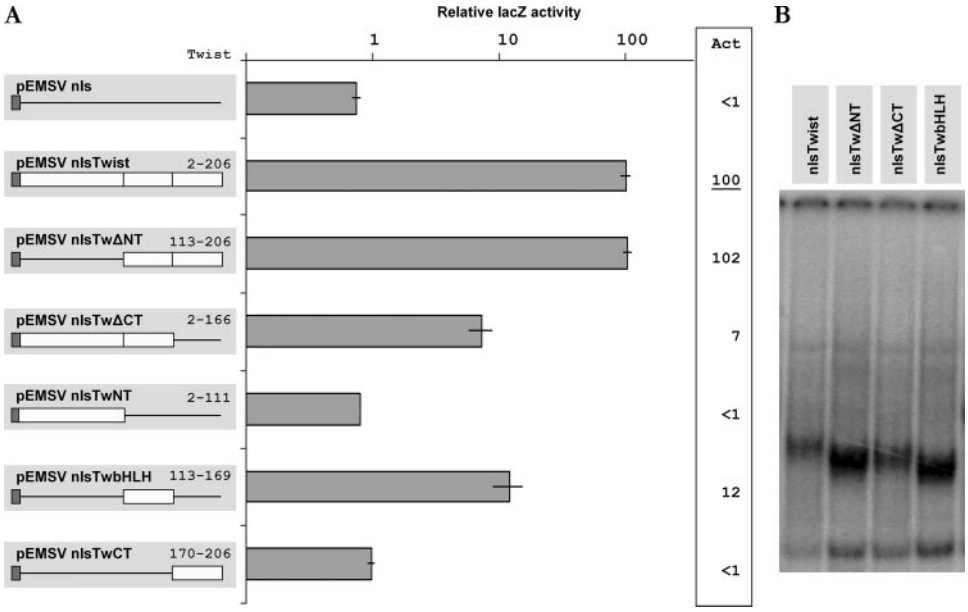

FIGURE 5. **The transactivation activity of Twist1 resides in the CT.** *A*, reporter activation (*Act*) after coexpression of E12 with full-length Twist1 or deletion constructs. When Twist1 is C-terminally truncated (nlsTwΔCT and nlsTwbHLH), transactivation is strongly reduced. In contrast, the N-terminally truncated mutant (nlsTwΔNT) displayed transactivation similar to full-length Twist1. Mutants lacking the bHLH domain (nlsTwCT and nlsTwNT) did not activate above background. Reporter activation is given in arbitrary units. Activity achieved by coexpression of Twist1 and E12 was set to 100% (note the logarithmic scale). The data are from a single representative experiment. *B*, all transactivating Twist1 mutants were able to dimerize with E12 and bind the E-box E3. Neither transcriptional activation nor DNA binding was observed in the absence of E12 (not shown).

TABLE 1

The activation domains of Twist1 and E12 are functionally independent

Twist1 deletion mutants were assayed for transcriptional activation in the presence of various E12 mutants. Independent of the mutation in the dimerization partner, the loss of the Twist1-AD results in an approximately 80% reduced activity, whereas the loss of the E12-AD2 results in an approximately 50% reduced activity. The Twist1-AD is thus more potent than the E12-AD2. Activity achieved by coexpression of Twist1 and E12 was set to 100%. Each activity measure represents an average of five independent experiments. The activity-measures were grouped according to ADs present: Twist1 and E12 (*a*), Twist1 (*b*), E12 (*c*), and no ADs (*d*). Analysis of variance analysis resulted in following *p* values: *a*, 0.48; *b*, 0.46; *c*, 0.85; *d*, 0.59; *a* and *b*, $3{\cdot}10^{-4}$; *b* and *c*, $2{\cdot}10^{-3}$; *c* and *d*, $6{\cdot}10^{-3}$.

Protein (amino acids)	E12 (217–653)	E12ΔNT (415–653)	$E12_{mut}AD2$ (217–653)
nlsTwist (2–206)	100_a	52_b	62_b
nlsTwΔNT (113–206)	130_a	39_b	53_b
nlsTwΔCT (2–166)	21_c	10_d	8_d
nlsTwbHLH (113–169)	24_c	9_d	12_d

may be explained by the presence of cellular E-proteins. Indeed, it was shown that cellular Twist1 forms DNA-binding complexes in a large size range, most likely by dimerization with different E-proteins (37). However, when compared with the transiently expressed proteins, the endogenous E-proteins are scarce, and the effect of these can thus be neglected.

It has been shown before that bHLH factors can be forced to dimerize by tethering with a flexible polypeptide linker and that tethered MyoD1/E47 dimers are resistant to Id challenge (69). However, even when two Twist1 monomers were joined in this way, no activity of homodimers was detected. In contrast, the tethered Twist1 "double monomers" interacted potently with E12 monomers. In gel retardation assays, these complexes produced band retardations, which indicate the formation of a tetrameric complex composed of two E12 monomers and one tethered Twist1 double monomer. The binding of two E12 factors to the tethered Twist1 double monomer confirms correct folding of the bHLH regions of both Twist1 factors. This makes it unlikely that incorrect folding is the explanation for the missing activity of tethered Twist1 homodimers. Another possible explanation for the absence of active Twist1 homodimers could be that the polypeptide linker does not allow intramolecular dimerization. However, we consider this unlikely, not only because similar linkers have been shown to be sufficiently flexible (69) but also because the same $(GGGS)_5$ linker allows intramolecular dimerization between Twist1 and E12 in either position. Also, the fact that E12∼Tw and Tw∼E12 both are functional in the reporter and the DNA binding assays proves that Twist1 and E12 can be functional in both positions, *i.e.* before and after the linker. We therefore conclude that the tethered dimers are fully functional. This implies that under our experimental conditions, the Twist1-E12 affinity must significantly exceed the Twist1 as well as the E12 homodimerization affinities.

When compared with the Twist1-E12 dimer and with the tethered E12∼Tw, the tethered Tw∼E12 complex displayed increased migration in the gel retardation assay. As a Western blot of a denaturing gel showed that the tethered protein has the expected size, the faster migration suggests an altered structure of the native Tw∼E12 protein. However, transactivation of the reporter and sequence-specific DNA binding did not indicate any functional difference between the two tethered heterodimers or a coexpression of the two monomers. Interestingly, increased migration of tethered heterodimers with E-proteins as C-terminal partners has also been observed by others with MyoD1 as well as Twist1 in N-terminal positions (57, 69). The N-terminal region of the E-protein, which is directly attached to the linker, may adopt an alternative conformation, thereby causing the faster migration.

The choice between homo- and heterodimerization may be subject to regulation. This is supported by observations that phosphorylation of Twist1, as well as of E47, is followed by a shift in the preferred dimerization partner (12, 77). Studies with Twist1 monomers and tethered Twist1 homodimers suggest, in addition, that an E12-independent transactivation by Twist1 may be enhanced by BMP7 (57). It is thus conceivable that formation of transactivating Twist1 homodimers can be induced under certain physiological conditions, yet in our experimental conditions, the tethering alone is insufficient to enforce functional homodimerization. The fact that Twist1 functioned as a transactivator even in the absence of E12-ADs

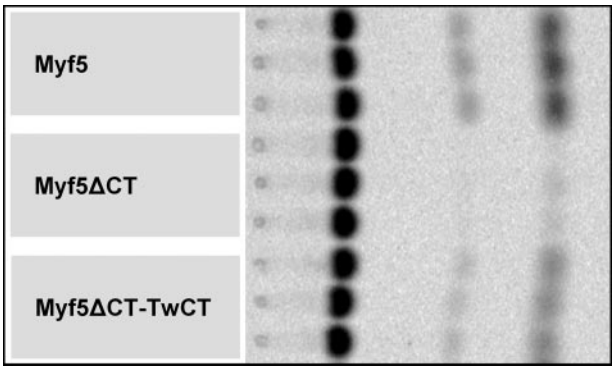

FIGURE 6. **The Twist1 CT retains transactivation properties in an unrelated context.** The transcriptional activation of the muscle-specific *Myf4* reporter by various constructs was assayed in the presence of E12. The Twist1 CT can functionally substitute for the known activation domain in the CT of Myf5. Full-length Myf5 served as a positive control for reporter transactivation. The data are from a single representative experiment.

TABLE 2

Three amino acids are essential for the Twist1 transactivation domain

Point mutations of Twist1 were assayed for transcriptional activation when coexpressed with E12. Transcriptional levels below 50% were considered to represent reduced transactivation, whereas levels above 75% were considered to represent full transactivation. Intermediate levels were considered inconclusive. According to this grouping, the values of mutant-transactivation in the last column are low (−), high (+), or intermediate (~), respectively. Activity achieved by coexpression of Twist1 and E12 was set to 100%. Each activity measure represents an average of three independent experiments.

Wild-type residue	Mutation	Activity		
		+	~	−
Cys-179	Gly	81		
Glu-185	Asp	185		
	Lys	88		
	Arg	93		
Arg-186	Ala	103		
	Lys	88		
Leu-187	Gly			36
Ser-188	Ala	102		
Phe-191	Pro			25
	Ala			29
	Gly			23
Ser-192	Ala	124		
	Pro		58	
Arg-195	Gly			33
	Glu		53	
Met-176/Cys-179	Ile/Gly	85		
Leu-187/Arg-195	Gly/Gly			21
Leu-187/Phe-191	Gly/Gly			25
Phe-191/Arg-195	Gly/Gly			24
Phe-191/Ser-192	Val/Pro			27
Leu-187/Phe-191/Arg-195	Gly/Gly/Gly			24
Arg-186/Ser-188/Phe-191	Leu/Gly/Leu			42

(Fig. 4*A*) argues that the Twist1-AD is functionally independent of the dimerization partner. We therefore expect that a transactivation mediated by Twist1 homodimers, as has been reported (57), would also rely on the here identified Twist1-AD.

The cooperative transactivation by Twist1 and E12 activation domains reported here potentially provides an additional level of regulation, where fine-tuning of the transcriptional activity is achieved by silencing of individual ADs. Whether such intricate regulation actually occurs remains to be established yet, the notion that MyoD1 transactivates certain reporters only when dimerized to truncated, but not full-length E12 (78), suggests that in the full-length complex, transactivation domains can be kept silent by properties of the most N-terminal region of E12.

Mammals	(Mm)	185	E	R	L	S	Y	A	F	S	V	W	R	M	E	197
Amphibians	(Xl)	145	E	R	L	S	Y	A	F	S	V	W	R	M	E	157
Fish	(Dr)	150	E	R	L	S	Y	A	F	S	V	W	R	M	E	162
Lancelets	(Bb)	169	E	R	L	S	Y	A	F	S	V	**S**	R	**Q**	E	180
Fly	(Dm)	470	E	**K**	L	S	Y	**L**	F	**G**	V	W	R	M	E	482
Arachnida	(At)	199	E	**L**	L	S	**R**	**E**	F	**L**	**F**	**R**	R	M	**Q**	211
Leeches	(Hr)	310	**D**	**K**	L	**G**	Y	A	F	S	V	W	R	M	E	322
Snails	(Io)	93	**D**	**C**	L	S	Y	A	F	S	V	W	R	M	E	105
Nematode	(Ce)	93	**Y**	**N**	L	**Q**	**S**	A	F	**N**	**M**	W	R	**G**	**N**	105
Jellyfish	(Pc)	125	E	R	L	S	Y	A	F	S	V	W	R	M	E	137

FIGURE 7. **Essential residues of the Twist1 transactivation domain are evolutionarily conserved.** Sequence alignment reveals that the amino acids that are essential for transcriptional activation by Twist1 are conserved throughout the animal kingdom. Non-conserved residues (shown in *bold*) are found throughout the region except in the positions of the essential residues Leu-187; Phe-191; Arg-195 (*boxed*). Species and gene library accession numbers are as follows; *Mm*, *Mus musculus* (GenBank accession number AAH83139); *Xl*, *Xenopus laevis* (GenBank accession number AAI29770); *Dr*, *Danio rerio* (GenBank accession number ABA08197); *Bb*, *Branchiostoma belcheri* (GenBank accession number AAD10038); *Dm*, *Drosophila melanogaster* (GenBank accession number CAA31024); *At*, *Achaearanea tepidariorum* (GenBank accession number BAD51393); *Hr*, *Helobdella robusta* (GenBank accession number AAL05567); *Io*, *Ilyanassa obsolete* (GenBank accession number AAG25636); *Ce*, *C. elegans* (GenBank accession number AAC26105); *Pc*, *Podocoryne carnea* (GenBank accession number CAC12667).

The CT of Twist1 is both necessary and sufficient for transcriptional activation, and in this respect, a CT-perturbed mutant may serve as a dominant negative factor either by sequestering E-proteins and/or by binding to E-box targets. Interestingly, the Twist1-CT was reported to be a target for proteolytic cleavage with subsequent degradation of the protein (79). We did not observe any reduced stability of the CT-truncated mutants and therefore suspect that the effect of a CT truncation may be context-dependent, possibly producing a dominant negative Twist1. Such a truncated protein would retain several inhibitory properties, for instance, the MyoD1 inhibitory function, which is mediated by the basic region (29), and the p300/CREB-binding protein inhibitory functions mediated by the NT region of Twist1 (30).

We identified residues Leu-187, Phe-191, and Arg-195 in the Twist1 WR domain as essential for transactivation. The specific conservation of these three residues (Fig. 7) indicates that Twist may function as a transactivator in all multicellular animals. The strictly preserved spacing of the residues (LX_3FX_3R) strongly points to a structural importance of this domain. In an α-helical structure suggested by Chou-Fasman analysis, the essential Leu-187, Phe-191, and Arg-195 residues would be in proximity and could thus function as a conserved epitope directly involved in transactivation (Fig. 8). The α-helical structure is known to occur in activation domains and has, for instance, been found in both AD1 and AD2 of $E12_{WT}$ (70, 80). The predicted α-helical structure of the WR domain is further supported by the fact that a S192A substitution had no effect on the Twist1 transactivation activity, whereas a S192P substitution, which will break a potential helical structure, significantly perturbed transactivation. The fact that the R195E substitution is less disturbing than the R195G substitution can in this model be explained by glutamine favoring the formation of α-helical structures more than glycine. In contrast, the F191A substitution is fully compatible with helix formation, yet the transactivation is impaired similar to when the entire CT is deleted, which demonstrates a function of Phe-191 beyond helix forma-

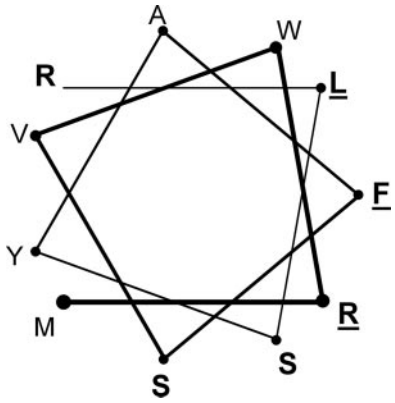

FIGURE 8. **The Twist1 transactivation domain is likely to adopt an α-helical structure.** When drawn into an α-helical structure as suggested by Chou-Fasman analysis, the residues essential for Twist1-mediated transactivation come into close proximity. The residues tested by mutagenesis are in *bold type*, and functionally important residues are *underlined*.

tion. In summary, we have presented evidence that the Twist1-AD localized in the WR domain adopts an α-helical structure, in which a conserved $(LX_3FX_3R)_\alpha$ epitope is essential for transactivation.

The majority of the transactivationally impaired mutants reported in this study have not been phenotypically evaluated in animals. Only the S192P mutation, which displayed a slightly impaired transactivation, has previously been associated with a phenotypic defect. This mutant was termed Charlie Chaplin due to a characteristic waltzing gait of heterozygous mice. Homozygous mutant mice died immediately after birth, displaying several phenotypic defects (53). On the protein level, the Charlie Chaplin mutation was associated with an impaired Runx2 binding, which weakens the Twist1-mediated inhibition of Runx2 (53). The reduced transactivation by the S192P mutant here presented makes it, however, possible that the Charlie Chaplin phenotype is caused by impaired transactivation. If this is the case, a similar yet more severe phenotype would be expected for a mouse harboring any of the mutations that mediate only weak transactivation. Alternatively, the Charlie Chaplin is the combined phenotype resulting from both impaired Runx2 inhibition and impaired transactivation. Potentially, the two mechanisms may be distinguished by comparing the phenotypic effects of specific mutations, which interfere only with Runx2 binding or with Twist1 transactivation. It could therefore be interesting to determine the Runx2 affinity of Twist1 bearing each of the point mutations reported here.

Our findings describe in detail the transactivation activity of a vertebrate Twist1 protein. The precise localization of the hitherto unknown Twist1 transactivation domain enables us to create mutants in which only the transcriptional activation by Twist1 is eliminated, whereas other activities such as transcriptional repression and inhibition of histone acetylation are likely to be maintained. It is presently unknown whether Twist1 is involved through its inhibitory or transactivating functions in pathological conditions such as the Saethre-Chotzen syndrome and tumor metastasis. Hopefully, the mutants reported here will aid in elucidating the functional role of Twist1 in these processes.

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