A dominant repression domain in Tbx3 mediates transcriptional repression and cell immortalization: relevance to mutations in Tbx3 that cause ulnar-mammary syndrome

Hanqian Carlson¹,², Sara Ota¹, Christine E. Campbell³ and Peter J. Hurlin¹,²,*

¹Shriners Hospitals for Children and ²Department of Cell and Developmental Biology, Oregon Health Sciences University, 3101 Sam Jackson Park Road, Portland, OR 97201, USA and ³Department for Cancer Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH 44195, USA

Received June 8, 2001; Revised and Accepted July 31, 2001

Mutations in Tbx3 are responsible for ulnar-mammary syndrome (UMS), an autosomal dominant disorder affecting limb, tooth, hair, apocrine gland and genital development. Tbx3 is a member of a family of transcription factors that share a highly conserved DNA-binding domain known as the T-domain. UMS-causing mutations in Tbx3 have been found at numerous sites within the TBX3 gene, with many occurring downstream from the N-terminally located T-domain. The occurrence of mutations downstream of the DNA-binding domain raises the possibility that there exist important functional domains in C-terminal portions of the Tbx3 protein that affect its behavior as a transcription factor. To determine if and how such C-terminal mutations affect transcription we have mapped regions that confer transcriptional activity and nuclear localization and characterized the DNA binding properties of Tbx3. We find that Tbx3 binds the canonical Brachyury binding site as a monomer and represses transcription. We show that a key repression domain (RD1) resides in the Tbx3 C-terminus that can function as a portable repression domain. Most UMS-associated C-terminal mutants lack the RD1 and exhibit decreased or loss of transcriptional repression activity. In addition, we identify a domain responsible for nuclear localization of Tbx3 and show that two C-terminal mutants of Tbx3 have increased rates of protein decay. Finally, we show that Tbx3 can immortalize primary embryo fibroblasts and that the RD1 repression domain is required for this activity. Our results identify critical functional domains within the Tbx3 protein and facilitate interpretation of the functional consequences of present and future UMS mutations.

INTRODUCTION

Members of the Tbx gene family play a variety of important roles during embryonic development, and mutations in at least two members of this family are responsible for congenital birth defects in humans (1). The hallmark feature of Tbx proteins is a highly conserved DNA-binding domain, the T-domain, first identified in Brachyury (2), the original member of the Tbx family. For Brachyury, it was shown that the T-domain mediates sequence-specific DNA binding as a monomer (2) and regions outside of the T-domain mediate transcriptional activation and repression functions (3). A subsequent study examining the crystal structure of the Xenopus Brachyury T-domain bound to DNA showed that the T-domain can also mediate homodimerization (4).

The brachyury gene was identified by positional cloning of the gene responsible for the classic mouse mutant called tailess or T (5). The tailess mouse has a very short tail caused by defective development of posterior mesoderm during gastrulation. The original tailess mouse was found to contain a large deletion that included a single allele of the brachyury gene, with the other allele apparently unaffected (5). Thus, haploinsufficiency of Brachyury appears to be responsible for the classical ‘tailess’ phenotype. Indeed, mice homozygous for a brachyury null allele survive only to midgestation and have more severe defects in posterior mesoderm, lack a notochord and die due to a failure of the allantois to extend and connect with the placenta (6). These results implicate brachyury gene dosage effects, and corresponding Brachyury protein levels as critical during development. Several additional mutant brachyury alleles have been identified that contain mutations predicted to cause C-terminal truncations that leave the T-domain DNA-binding region intact (7). Importantly, these mutations result in a more severe heterozygous phenotype than null alleles, causing complete loss of the tail and skeletal abnormalities (7). These results suggest that C-terminal frameshift and termination mutations in brachyury lead to the production of mutant proteins that have dominant-negative activity. Such proteins are expected to bind their target genes, but misregulate expression due to disruption of regions critical
for proper transcriptional regulation. Consistent with this idea, C-terminal truncation mutants of Brachyury that leave the DNA-binding domain intact behave as dominant-negative proteins when expressed in Xenopus embryos (8).

Mutations in the brachyury-related gene TBX3 were recently identified as responsible for the autosomal dominant human disease syndrome ulnar-mammary syndrome (UMS) (9). UMS is a pleiomorphic syndrome characterized by malformations of posterior elements of the upper limb including the ulnar, metacarpals and phalanges as well as apocrine, dental and urogenital abnormalities (10). Like Holt–Oram syndrome caused by mutations in TBX5 (11–13) and the mouse ‘tailless’ phenotype caused by brachyury mutations (5,7), UMS is a haploinsufficiency syndrome; only a single TBX3 allele is mutated. Thus, the behavior of cells expressing these genes appears extremely sensitive to their expression level. The original description of TBX3 mutations found in individuals with UMS (9) indicated that disease-associated mutations either eliminated or disrupted the T-domain and DNA binding. These results suggested that mutations that destroyed the T-domain generated a single null allele of TBX3, and thus strict haploinsufficiency (reduced gene dosage) of Tbx3 was responsible for UMS (9). However, several additional single allele TBX3 mutations were subsequently identified in a second, more extensive set of patients with UMS that mapped to regions downstream of the T-domain, including ones predicted to cause only small C-terminal truncations, leaving the T-domain intact (14). The range of morphological abnormalities in individuals containing these latter mutations is quite similar to those containing mutations that eliminate the T-domain (14), raising the possibility that mutations downstream of the T-domain may disrupt critical activities of Tbx3.

In this study, we investigate molecular and biochemical mechanisms that regulate Tbx3 function and how these are affected by C-terminal truncation mutations associated with UMS. In addition, we show that Tbx3 can immortalize primary cells and that this activity is dependent on a dominant repression domain located in the Tbx3 C-terminus. Our results indicate that loss or disruption of C-terminal regions of Tbx3 has severe consequences for the normal function of Tbx3.

RESULTS

Transcriptional activity of Tbx3 and identification of a critical repression domain

To determine the transcriptional activity of Tbx3 and define regions of Tbx3 that contribute to transcriptional regulation, fusion proteins were made between the DNA-binding domain of Gal4 (Gal4DB) and either wild-type (WT) Tbx3 (accession no. NM0055996) or portions of the Tbx3 protein. These fusion proteins were expressed in HEK293 cells and transcriptional activity measured using a luciferase reporter gene containing four promoter-proximal Gal4 binding sites (Fig. 1A). Western blot analysis of the various Gal4 fusion proteins in transfected cells indicated similar expression levels (not shown). Consistent with a previous study examining a putative Xenopus Tbx3 ortholog ET (15), we found that Gal4-Tbx3 repressed transcription. Furthermore, several regions within the Tbx3 protein conferred transcriptional repression as well as transcriptional activation (Fig. 1A). Of particular interest was the region that included amino acids 567–623, which repressed transcription to levels near that of the wild-type protein and showed a dominant repression activity when fused with an adjacent region of Tbx3 (amino acids 423–500) that activated transcription (Fig. 1A). The presence of a key repression domain specifically in this region of Tbx3 is consistent with results of transcription assays using C-terminal truncation mutants in the context of binding to a consensus Brachyury site (see below).

To determine whether Tbx3 amino acids 567–623 can function as a transferable repression domain, we fused this region to the potent transcriptional activation domain of VP16 (Fig. 1B). Similar to the ability of this region to override an adjacent activation domain in the Tbx3 protein (Fig. 1A), it showed dominant repression activity over activation by VP16. Other regions of Tbx3, including amino acids 100–200, which showed repression activity as a Gal4 fusion, failed to affect activation by VP16 (Fig. 1A and data not shown). As shown in Figure 1C, expression levels for these VP16 fusion proteins were comparable.

DNA binding of Tbx3 and C-terminal UMS-associated mutant proteins

The T-domain of Tbx proteins mediates DNA binding and there is evidence showing that this domain may also mediate homodimerization (4). Thus, mutations found in Tbx3 that truncate the protein before or within the T-domain, as well as mutations that abrogate DNA binding, are thought to function as null alleles. Indeed, characterization of the DNA binding properties of Tbx3 proteins with engineered UMS mutations that fall within the T-domain disrupt DNA binding (C.E.Campbell, personal communication). However, it is more difficult to explain why mutations predicted to truncate Tbx3 downstream of the T-domain also act as null alleles. One possibility is that C-terminal regions directly or indirectly effect DNA binding or dimerization by the T-domain. To test this possibility we have constructed a series of expression plasmids containing mutant Tbx3 cDNAs based on mutations associated with UMS (Fig. 2A). We also constructed plasmids encoding non-UMS-associated Tbx3 truncations to assist in analyzing DNA binding and transcriptional function (Fig. 2A).

To examine DNA binding we prepared whole cell extracts from HEK293 cells transfected with wild-type Tbx3 and the various Tbx3 truncation mutants constructed. Electrophoretic mobility shift assays (EMSAs) were performed by mixing equal amounts of nuclear extract with a radiolabeled oligonucleotide containing the consensus Brachyury binding site, ATTTCACACCTAGGTGTGAAAT (2). As shown in Figure 2C, each of the truncation mutants was capable of binding DNA, although differences in the amount of protein–DNA complexes suggest that some differences in DNA binding properties might exist. In addition, all of the truncation proteins with the exception of Tbx3-605 and -652 produced a second slower mobility shift, raising the possibility that the more severe C-terminal mutant proteins might bind DNA as both monomers and homodimers. To test for the ability of wild-type Tbx3 to form homodimers in cells, we performed EMSAs with cell extracts made from cells cotransfected with C-terminal truncation mutants and wild-type Tbx3 (Fig. 2C, right side). If Tbx3 binds DNA as a dimer then intermediate
mobility shifts consisting of wild-type and mutant Tbx3 would be detected in this assay. Consistent with results using in vitro translated proteins (not shown), no intermediate size mobility shifts were produced compared with the mobility shifts of the individual proteins (Fig. 2C). These results suggest that wild-type Tbx3 binds the preferred palindromic Brachyury binding site as a monomer and does not dimerize with C-terminal truncated forms. However, experiments designed to determine whether C-terminal truncation mutants are capable of binding DNA as both monomers and homodimers have been inconclusive (not shown).

Transcriptional activity of UMS-associated C-terminal mutants

Since the C-terminal truncation mutants of Tbx3 tested all bound DNA we next focused on whether disrupted or
Figure 2. DNA binding of Tbx3 and C-terminal UMS mutant proteins to the preferred palindromic Brachyury binding site. (A) Schematic diagram of the structure of the Tbx3 gene showing exons (boxed regions) based on the work of Bamshad et al. (14). Gray boxes denote the exons encoding the T-domain (14). The position of mutations identified by Bamshad et al. (14) downstream from the T-box are indicated with numbers representing the size in amino acids of Tbx3 truncation mutants predicted to be produced by the corresponding mutation. Please note that the Tbx3 cDNA used in this study does not contain the alternatively spliced exon 2a (14). WT denotes wild-type proteins and asterisks denote non-UMS associated mutant proteins used in this study. (B) Autoradiograph showing expression of the indicated proteins in HEK293 cells by western blot using an anti HA-antibody (proteins are HA-tagged at the 3′ end). (C) EMSA of whole cell extracts of wild-type Tbx3 (Tbx3-WT) and Tbx3 truncation mutants to a radiolabeled oligonucleotide containing the Brachyury binding site AA TTTCA CACCTAGGTGTA GTTG AAAAT. The left side of the panel shows EMSA with wild-type Tbx3 with 100× unlabeled Brachyury oligonucleotide demonstrating specificity of the Tbx3-DNA complex and with anti-HA (α-HA) showing supershift of the Tbx3-DNA complex. The right side of the panel shows EMSA of cells cotransfected with Tbx3-WT and the indicated plasmids expressing truncated proteins. Extracts used were made from equal numbers of transfected cells. ‘Empty’ represents cells transfected with empty plasmid and ‘cells’ represent untransfected cells.
abrogated transcriptional function associated with loss or disruption of the C-terminal repression domain might be a property of these forms. We first measured the transcriptional activities of wild-type Tbx3, C-terminal UMS-associated mutant forms and a form lacking the entire C-terminus downstream from the T-domain (Tbx-300), using an engineered luciferase reporter plasmid containing two copies of the Brachyury binding site at a promoter-proximal position (16). Tbx3 repressed transcription approximately 3- to 5-fold relative to empty expression vector (Fig. 3). A range of transcriptional activity was seen with the different C-terminal mutants: the most extensive mutant forms (Tbx3-300, Tbx3-343 and Tbx3-360) were transcriptionally inert, Tbx3-433 and Tbx3-605 repressed transcription less effectively than wild-type Tbx3, and Tbx3-652 repressed transcription similarly to the wild-type protein (Tbx3-WT, Fig. 3). Although these results are consistent with our Gal4 fusion protein studies showing that a critical transcriptional repression domain exists in the Tbx3 C-terminus (Fig. 1), the ability of Tbx3-652 to repress transcription similarly to wild-type Tbx3 indicates that loss of this domain and transcriptional repression is not an absolute requirement for UMS.

Protein stability and subcellular distribution of Tbx3 and C-terminal mutant Tbx3 proteins

Since loss of transcription repression potential is likely not by itself responsible for UMS, we examined whether our C-terminal truncation proteins exhibited altered protein stability properties and/or altered subcellular localization that might be related to UMS. To determine whether sequence within the C-terminus affected Tbx3 protein turnover, we used a pulse–chase analysis of wild-type Tbx3, Tbx3-300, Tbx3-343 and Tbx3-360) were transcriptionally inert, Tbx3-343 and Tbx3-605 repressed transcription less effectively than wild-type Tbx3, and Tbx3-652 repressed transcription similarly to the wild-type protein (Tbx3-WT, Fig. 3). Although these results are consistent with our Gal4 fusion protein studies showing that a critical transcriptional repression domain exists in the Tbx3 C-terminus (Fig. 1), the ability of Tbx3-652 to repress transcription similarly to wild-type Tbx3 indicates that loss of this domain and transcriptional repression is not an absolute requirement for UMS.

To examine the subcellular distribution of Tbx3 and C-terminal mutants, HA-tagged proteins were expressed in HEK293 cells and detected by immunofluorescence. As shown in Figure 5, wild-type Tbx3 protein (Tbx3-WT, panels D–F), and Tbx3-300 (panels G–I) localized to the nucleus (yellow signal in merged image). Similarly, other Tbx3 mutant forms lacking more distal regions of the C-terminus also maintained a wild-type distribution pattern (not shown). Thus, mutations that remove nearly all sequence C-terminal to the T-domain do not cause mislocalization of Tbx3. To identify region(s) required to localize Tbx3 to the nucleus, the N-terminal 300 amino acids were searched for stretches of basic amino acids that might typically serve as nuclear localization signals. A cluster of basic amino acids was identified at amino acid positions 292–297 (RREKRK). Deletion of this site in the context of the wild-type Tbx3 protein (creating Tbx3∆NLS) caused mislocalization to a perinuclear site and to the cytoplasm (Fig. 5J–L). Furthermore, truncation mutants of Tbx3 that lack this site showed a similar subcellular distribution to the 292–297 deletion (not shown), therefore we conclude that the RREKRK sequence (or sequence within) functions as the Tbx3 nuclear localization signal.
Immortalization of primary mouse embryo fibroblasts by Tbx3, but not a mutant lacking RD1

It was recently demonstrated that Tbx2, which is closely related to Tbx3 (17), is capable of immortalizing mouse embryo fibroblasts (MEFs) when ectopically expressed in these cells (18). This result prompted us to test the ability of Tbx3 to immortalize MEFs. Two protocols were used: first, primary MEFs at passage three were transfected with retroviral vectors expressing either wild-type Tbx3 or a mutant containing a deletion in the RD1 (amino acids 555–605). Transfected cells were selected with G418 for 3 weeks and the number of colonies surviving determined. Cells transfected with wild-type Tbx3, but not Tbx3-605, bind only as monomers. Furthermore, our DNA binding assays demonstrate an inability of wild-type Tbx3 to interact with the C-terminal truncation mutants, raising the possibility that dimers between wild-type Tbx3 and C-terminal truncation mutants are capable of binding the palindromic Brachury binding site (Fig. 2). Although we cannot rule out this activity is mediated by the RD1.

DISCUSSION

The Tbx family of proteins has emerged as key transcriptional regulators of cell fate decisions during embryonic development. In humans, mutations in Tbx3 that cause UMS (9,14) have illuminated the role of this protein in patterning posterior elements of the upper limb and in regulating mammary, apocrine gland, tooth and sexual development. Mutations that cause UMS occur throughout the Tbx3 gene, including regions of the N-terminus critical for the DNA-binding domain (the T-domain) and regions of the C-terminus of unknown function (9,13). Although UMS mutations in the T-box that disrupt the ability of Tbx3 to bind DNA are expected to be null, loss of function alleles, it is less clear how mutations that effect only C-terminal regions are involved in disease. Thus the aim of this study was to determine the transcriptional activities of Tbx3, how these activities are regulated in cells and to identify domains in the Tbx3 protein critical to its function and that might be disrupted as a result of UMS mutations. Since the engineered mutant Tbx3 proteins used in this study do not include unique sequence additions predicted to result from frameshift mutations in the Tbx3 gene, we cannot formally rule out that such sequence additions may affect Tbx3 function. Nonetheless our series of UMS-based mutants (12,14), and the cell systems used in our analysis allow for an approximation, if not an accurate assessment, of the activity of the predicted mutant proteins.

Regulation of transcription by Tbx3

Although the T-domain is highly conserved amongst members of the Tbx family, it is not clear whether the subtle differences that exist reflect significantly different biochemical properties. Our results with Tbx3 indicate that all UMS-associated C-terminal truncation mutants are capable of binding the palindromic Brachury binding site (Fig. 2). Although we cannot rule out that, under the DNA binding conditions used, some of the truncation mutants can bind the Brachury site as dimers, our results suggest that wild-type Tbx3, as well as Tbx3-605 and Tbx3-652, bind only as monomers. Furthermore, our DNA binding assays demonstrate an inability of wild-type Tbx3 to interact with the C-terminal truncation mutants, raising the possibility that dimers between wild-type Tbx3 and C-terminal truncation mutants do not occur in UMS patients. However, the ability of C-terminal truncation mutants to bind DNA predicts that these proteins, if produced in vivo, could compete with wild-type proteins for binding to DNA.

A previous study by He et al. (15) showed that the Xenopus Tbx3 ortholog, ET, repressed transcription as a Gal4 fusion protein. We have confirmed and extended these results by...
showing that human Tbx3 is a transcriptional repressor both in
the context of a Gal4 fusion protein binding to Gal4 binding
sites and in the more natural setting of wild-type Tbx3 binding
to Tbx binding sites. Although Tbx3 shows net transcriptional
repression activity, our Gal4 fusion study shows that there are
several regions within the Tbx3 protein that have both repres-
sion and activation activity. Although a region capable of
activating transcription was identified in Tbx3 (Fig. 1), it is
unclear whether this activity is relevant to Tbx3 function. This
region was capable of activating transcription only when
isolated from other regions of Tbx3 (Fig. 1A), indicating that it
is subordinate to repression by Tbx3. Indeed, our Gal4 studies
showed that an adjacent repression domain (RD1) located
between amino acids 567 and 623 is dominant over this
activating region in the context of the Tbx3 protein. Further-
more, the RD1 functioned as a portable dominant repression
domain when fused to the potent activation domain of VP16
(Fig. 1B). Thus, although the net repression activity of

Figure 5. Subcellular distribution of Tbx3 and C-terminal truncation mutants and mapping of the Tbx3 nuclear localization domain. HA-tagged proteins were
expressed in HEK293 cells and examined by immunofluorescence using an anti-HA antibody and FITC-conjugated secondary antibody. (A, D, G and J) The intra-
cellular localization of Tbx3 proteins (green signal); (B, E, H and K) the corresponding propidium iodide-stained cells in the field (red fluorescence); and (C, F, I
and L) the merged images. Wild-type Tbx3 (D–F) and Tbx3-300 (G–I), a truncation form of Tbx3 containing the N-terminal 300 amino acids of Tbx3, localize to
the nucleus. Tbx3-ΔNLS, a mutant containing a deletion of amino acids 292–298 (J–L) results in localization primarily to the cytoplasm. (M–O) Colocalization
of Tbx3ΔNLS-292–298 with actin in the cytoplasm. (M) FITC-conjugated secondary antibody staining of the Tbx3ΔNLS protein. (N) Rhodamine-conjugated secondary
antibody staining of anti-actin, and (O) merged image.
Tbx3 appears relatively weak (3–5-fold), our results support the conclusion that transcriptional repression mediated by Tbx3 is dominant over the activity of transcriptional activators. It will now be important to determine the mechanism by which Tbx3 and the RD1 mediate repression and whether auxiliary factors are capable of mediating and/or modulating this activity.

Mutant Tbx3 proteins and UMS
Studies of the relationship between the phenotype of different tailess mouse mutants and the nature of mutations in the brachyury gene have demonstrated that C-terminal truncations downstream of the T-domain generate proteins that have dominant-negative activity and produce phenotypes more severe than null alleles (7,8). Given the ability of each of the C-terminal mutant Tbx3 proteins to bind the Brachyury target sequence (Fig. 2), they might be expected, if produced in cells, to alter or block the normal regulation of Tbx3 target genes and yield unique, and potentially more severe, phenotypic consequences. However, in contrast to brachyury mutations, the genotype/phenotype relationship between mutations in Tbx3 and UMS suggests that true null alleles or putative null alleles (ones predicted to disrupt the T-domain) are equivalent to mutations predicted to disrupt regions downstream of

Figure 6. Tbx3 allows primary MEFs to escape senescence. MEFs at passage three were transfected with empty RF retrovirus, RF-Tbx3RD1 or RF-Tbx3. Cells were grown in growth medium supplemented with G418 to select for transfectants (neomycin gene provided by the RF virus) for 3 weeks, then stained with crystal violet. Whereas only small abortive colonies were produced from empty virus and RF-Tbx3RD1 transfected cells, many large colonies were produced from cells transfected with Tbx3. This experiment has been repeated twice with essentially identical results. (B) Western blot of empty RF, RF-Tbx3RD1 and RF-Tbx3 virus-infected MEFs to demonstrate viral expression of Tbx3RD1 and Tbx3 proteins. Growth curve of MEFs infected with empty virus and Tbx3 virus. Cells were passaged every 3.5 days according to a modified 3T3 protocol (18).
the T-domain (9,14). An important consideration, however, is that C-terminal truncation mutants of Tbx3 (but not Brachyury or Tbx5) may not be produced in cells, or may be produced at reduced levels due to nonsense-mediated mRNA decay (19). This latter situation appears to apply to certain disease-causing mutations, including ones in the fibrillin gene that cause Marfans syndrome (19). Another possibility is that C-terminal truncation mutants of Tbx3 are expressed, but that regions affecting protein turnover are lost, resulting in lower protein levels in cells and UMS. Indeed, we find that Tbx3-300 and Tbx3-652 degraded at a slightly increased rate compared with the wild-type protein (Fig. 4). Although it is not known whether the decreased turnover rates are significant, since haploinsufficiency of Tbx3 is postulated to cause UMS, it is certainly possible that many of the cells that express Tbx3 are sensitive to minor changes in its expression. However, since Tbx-652 retains wild-type transcriptional repression activity (Fig. 3), a decrease in its steady-state protein levels might be expected to be less severe than for other mutations that appear transcriptionally inert. This does not appear to be the case for individuals containing the 1857 delC mutation (our Tbx3-652 mutant is modeled after this mutation) (14). Thus the lack of a clear genotype/phenotype relationship in UMS, together with our molecular studies, is consistent with an alternative mechanism, such as nonsense-mediated mRNA decay, as an important underlying mechanism in UMS.

A role for Tbx3 in pathways controlling cell senescence

The Tbx2 gene was recently shown to allow bypass of the premature senescence phenotype of mouse embryo fibroblasts that lack the polycomb group gene Bmi1 (18). Tbx2 is closely related to Tbx3 (17) and is also a transcriptional repressor (20). A potential mechanism allowing Tbx2 to immortalize cells is direct repression of the gene encoding p19ARF (18), an inhibitor of MDM2-mediated degradation of p53 (21). Although we are currently examining a potential role for p19ARF in immortalization by Tbx3, we do find that immortalization is dependent on the RD1 repression domain (Fig. 6). Translating this result to a potential in vivo function, expression of Tbx3 might be predicted to target cells to undergo additional rounds of cell division in an otherwise growth/proliferation suppressive environment. Given the abnormalities associated with mutations in Tbx3, one can speculate that cells targeted to proliferate by Tbx3 may be concomitantly or subsequently sensitized to patterning or inductive signals critical for proper development. Conversely, Tbx3 expression and its cell proliferation signal may be the target of patterning/inductive signals. Both of these scenarios are consistent with the fact that mutations in Tbx3 cause hypoplasia of several different tissues as well as patterning defects. Understanding the molecular mechanisms underlying the ability of Tbx3 to immortalize cells in culture and how this activity is manifest in vivo will likely lead to a better understanding of how mutations in Tbx3 cause UMS.

MATERIALS AND METHODS

Cell culture and DNA transfection and retroviral infection

HEK293 and mouse fibroblast NIH3T3 cells were obtained from American Type Culture Collection (ATCC). HEK293 cells were grown in 1× low glucose Dulbecco’s modified Eagle’s medium (DMEM) (Gibco) supplemented with 5% fetal bovine serum (FBS, Hyclone Laboratories, Logan, UT) and 1% penicillin–streptomycin (P/S, Gibco). Primary MEFs were isolated from embryonic day 13.5 mouse (C57Bl/6) embryos and grown in DMEM supplemented with 10% FBS. Colony formation by MEFs transfected with control and Tbx3 retroviral vectors was determined by staining cells with crystal violet. Experiments were performed in duplicate and repeated twice using MEFs isolated from different mice. For lifespan analysis, infected MEFs were plated at 4 × 10⁵ cells and passed every 3.5 days according to the modified 3T3 protocol described by Jacobs et al. (19). All cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO₂. Transfections were performed by the BES-buffered saline (BBS) calcium phosphate method (22). Retroviral supernatants were generated from transfected Phoenix cells and retroviral infections of MEFs were carried out for 24 h in DMEM/10% FBS. Following infection, MEFs were washed with phosphate-buffered saline (PBS) and fresh medium was added.

Expression plasmids

The human Tbx3 cDNA (accession no. NM005996) was cloned by RT–PCR from the human breast carcinoma cell line MDA Mbyte 453. Gal4–Tbx3 fusions were constructed by inserting different PCR-generated Tbx3 fragments with the Gal4 DNA-binding domain (amino acids 1–147) in the plasmid pSG424 (23). pcS2Tbx3 constructs were made by inserting PCR-generated fragments of full length or truncated versions of Tbx3 into BamHI/Xhol sites of pCS2 (D. Turner and R.Rupp, unpublished data). HA epitope-tagged Tbx3 sequences were generated by PCR using a 3’ primer encoding the HA epitope into the Xhol site of pcS2. All inserts were verified by sequencing. Retrovirus expression constructs Raf-Tbx3 and Tbx3ΔRD1 contain wild-type Tbx3 and Tbx3 containing a deletion of sequence coding for amino acids 555–605, respectively, in the RF retrovirus (Stratagene).

Luciferase assays

For Gal4 fusion experiments, 4 × 10⁵ HEK293 cells/well in 6-well dishes were transfected overnight with 0.5 µg of pGL24 × Gal4 luciferase reporter plasmid containing a 4-fold reiteration of the Gal4 recognition sequence in pGL2 (Promega), 0.5 µg of pCMVβGal and 2 µg of pSG424 fusion plasmid. For experiments using pcS2 expression plasmids, 0.5 µg of pGL2T₂ reporter plasmid (16), 0.5 µg of βGal and 2 µg of pCS2Tbx3 plasmid were used. Cells were harvested 48 h after transfection, washed with PBS, lysed with 100 mM potassium phosphate pH 7.8, 0.2% Triton X-100, 0.5 mM dithiothreitol (DTT). Lysates were centrifuged for 2 min at 14 000 r.p.m. and supernatants were assayed for luciferase and β-galactosidase activity using luciferin and Galacton Plus substrates, respectively, and a Tropix TR717 luminometer (Tropix, Massachusetts). β-Galactosidase activity was used as a control for transfection efficiency. Experiments were performed in triplicate and repeated three or more times.
Western blot and immunoprecipitation analysis

For transfected cells, cells were harvested 48 h after transfection and lysed with NP-40 buffer [150 mM NaCl, 20 mM Tris-HCl pH 7.8, 1 mM EDTA, 1% NP-40, 10% glycerol, and 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 µg/ml leupeptin added fresh]. Equal amounts of protein were resolved on 12.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were incubated in TTBS (10 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk overnight at 4°C. Membranes were then incubated for 1 h with 0.2 µg/ml rabbit polyclonal Ga4 antibody (Santa Cruz Biotechnology) or 0.2 µg/ml mouse monoclonal anti-HA antibody (Sigma). Membranes were washed with TTBS prior to incubation with alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (New England BioLabs). Immuno complexes were visualized using CDP-Star chemiluminescence reagents (Bio Labs) and exposure to X-ray film.

For immunoprecipitation, transfected 293 cells or NIH3T3 cells (1 × 10^6 cells/6-well dish) were washed twice with labeling medium [Met- and Cys-free DMEM (Gibco), dialyzed 10% fetal bovine serum (Gibco), 25 mM HEPES pH 7.4]. After washing, cells were starved in 1 ml labeling medium for 15 min at 37°C (5% CO₂) followed by labeling for 30 min in 1 ml labeling medium containing [³⁵S]methionine (Express label) 100 Ci/ml (Carbon-14). Membranes were resolved in 4.5% polyacrylamide gels (Tris/Borate/EDTA buffer). Gels were dried and exposed to film for 16–24 h.

Cell extracts and EMSAs

For transfected cells, cells were harvested 48 h after transfection and lysed with NP-40 buffer [150 mM NaCl, 20 mM Tris-HCl pH 7.8, 1 mM EDTA, 1% NP-40, 10% glycerol, and 10 µg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 25 µg/ml leupeptin added fresh]. Equal amounts of protein were resolved on 12.5% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes (Millipore Corp., Bedford, MA). Membranes were incubated in TTBS (10 mM Tris pH 7.6, 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat dry milk overnight at 4°C. Membranes were then incubated for 1 h with 0.2 µg/ml rabbit polyclonal Ga4 antibody (Santa Cruz Biotechnology) or 0.2 µg/ml mouse monoclonal anti-HA antibody (Sigma). Membranes were washed with TTBS prior to incubation with alkaline phosphatase-conjugated anti-rabbit IgG secondary antibody (New England BioLabs). Immuno complexes were visualized using CDP-Star chemiluminescence reagents (Bio Labs) and exposure to X-ray film.

For immunoprecipitations, transfected 293 cells or NIH3T3 cells (1 × 10^6 cells/6-well dish) were washed twice with labeling medium [Met- and Cys-free DMEM (Gibco), dialyzed 10% fetal bovine serum (Gibco), 25 mM HEPES pH 7.4]. After washing, cells were starved in 1 ml labeling medium for 15 min at 37°C (5% CO₂) followed by labeling for 30 min in 1 ml labeling medium containing [³⁵S]methionine (Express label) 100 Ci/ml (Carbon-14). Membranes were resolved in 4.5% polyacrylamide gels (Tris/Borate/EDTA buffer). Gels were dried and exposed to film for 16–24 h.

Cell extracts and EMSAs

A modified version of a previously described protocol (24) was used to make cell extracts for EMSAs. Briefly, transfected HEK293 cells in 6-well dishes were washed with PBS, harvested in 100 µl of F-buffer [10 mM Tris pH 7.05, 50 mM NaCl, 30 mM sodium pyrophosphate, 1% Triton X-100 and 1 tablet/10 µl protease inhibit cocktail tablet (Boehringer Mannheim)] per well. Cells were incubated on ice for 10 min, transferred to a tube and vortexed for 45 s and cleared by centrifugation at 14 000 r.p.m. A duplex oligonucleotide corresponding to the Brachyury consensus binding site [AATTTCACACCTAGGTGTGAAATT] containing 3 overhanging guanine residues was radiolabeled with [α-³²P]dCTP and Klenow (NEB). Labeled probe was isolated by Microspin G-50 columns (Amersham Pharmacia). Three microliters of cell extract, corresponding to equal numbers of starting cells, in a total volume of 23 µl [17 µl incubation buffer (10 mM Tris pH 7.8, 50 mM NaCl, 5 mM EDTA pH 8, 5% glycerol), 2 µl [³²P]oligonucleotide (20 000 c.p.m.) and 1 µl (5 µg) of poly dI dC (Amersham Pharmacia Biotech)], were incubated at 4°C for 25 min. Binding complexes were resolved in 4.5% polyacrylamide gels (Tris/Borate/EDTA buffer). Gels were dried and exposed to film for 16–24 h.

Immunofluorescence

HEK293 cells were plated 1 × 10⁵ cells/chamber into two-chamber slides. Forty-eight hours later, cells were fixed in ice-cold acetone for 10 min then washed with PBS for 5 min. The cells were incubated with polyclonal rat anti-HA (Sigma, 10 µg/ml) for 3 h at room temperature in a humidified chamber. Slides were then washed with PBS three times for 5 min, and then labeled for 30 min with anti-rat FITC-anti-IgG (1:32) secondary antibody. After washing three more times with PBS, the slides were stained for 10 min with propidium iodide. Slides were then washed twice for 10 min with PBS. 90% glycerol–PBS was used for mounting the slides. The slides were viewed with a Nikon eclipse E800 microscope. For actin staining, rabbit polyclonal anti-actin antibody (Sigma) and sheep anti-rabbit IgG conjugated to rhodamine (Sigma) were used.

ACKNOWLEDGEMENTS

We thank D. Turner for plasmid pCS2 and M. Ptashne for plasmid SG424. This work was supported by a grant from Shriners Hospitals for Children to P.J.H. and NIH grant DK48796 to C.E.C.

NOTE ADDED IN PROOF

We have now examined the lifespan of several different strains of primary MEFs following introduction of wild-type and different mutant Tbx3 proteins. We find that some mutant proteins lacking the Tbx3 C-terminus and the RD1 are not absolutely required for the immortalization. These results suggest that the Tbx3 C-terminus and the potential of Tbx3.

REFERENCES


