The Sry-related protein Sox10 is selectively expressed in neural crest cells during early stages of development and in glial cells of the peripheral and central nervous systems during late development and in the adult. Mutation of the Sox10 gene leads to neural crest defects in the Dominant megacolon mouse mutant and to combined Waardenburg-Hirschsprung syndrome in humans. Here, we have studied the four Sox10 mutations found to date in Waardenburg-Hirschsprung patients both in the context of the rat and the human cDNA. Unlike the rat Sox10 protein, which failed to show transcriptional activity on its own, human Sox10 displayed a weak, but reproducible, activity as a transcriptional activator. All mutant Sox10 proteins, including the one that only lacked the 106 last amino acids were deficient in this capacity, indicating that the carboxyl terminus of human Sox10 carries a transactivation domain. Whereas all four mutants failed to transactivate, only two failed to synergistically enhance the activity of other transcription factors. Synergy required both the ability to bind to DNA and a region in the amino-terminal part of Sox10. Those mutants that failed to synergize were unable to bind to DNA. Analysis of the naturally occurring Sox10 mutations not only helps to dissect Sox10 structure, but also allows limited predictions on the severity of the disease.

The family of Sox proteins is an evolutionary conserved group of transcription factors with diverse functions during development (1). All members of this family contain as their DNA-binding domain a variant form of the HMG1 box (2) that was first described in the sex-determining factor SRY (3). This HMG box contains three a-helical regions and is arranged in a twisted L shape. It presents its concave surface to the minor groove of DNA, thus allowing sequence-specific recognition. Binding of the HMG box induces a large conformational change in the DNA, thus allowing sequence-specific recognition. This conformational change is already expressed in the emerging neural crest. Sox10 gene family (6). During late development and in the adult, Sox10 expression was primarily detected in glial cells of the peripheral and the central nervous system. During early development, before the establishment of this “late” expression pattern, Sox10 is already expressed in the emerging neural crest. The neural crest gives rise to a number of different cell types and tissues, which include cells of the peripheral and the enteric nervous system as well as melanocytes. Accordingly, mutation of one Sox10 allele in the naturally occurring Dominant megacolon (Dom) mouse led to aganglionosis of the colon combined with pigmentation defects (7, 8). Mutation of both alleles is embryonic lethal and is characterized by severe defects in various parts of the peripheral and the enteric nervous system.

Heterozygous Sox10 mutations have also been detected in three sporadic and one familial case of the human Shah-Waardenburg syndrome (9). This disease combines the classical features of Waardenburg syndrome (deafness and pigmentation defects) with the aganglionic megacolon of Hirschsprung disease and thus bears resemblance to the phenotype of the Dom mouse (10). Here, we have performed an in vitro study of these four mutations and have analyzed their impact on Sox10 structure and function.

EXPERIMENTAL PROCEDURES

Plasmids—pCMV/rnSox10 contained rat Sox10 sequences (position 553–3003 according to GenBank™/EBI accession number AJ001029). pCMV/huSox10 carried the human Sox10 cDNA (GenBank™/EBI accession number AJ001183). Both Sox10 sequences were inserted between HindIII and BamHI sites of pCMV5. The previously identified Sox10 mutations WS029, 095, 059, and MIC were introduced into both cDNAs by polymerase chain reaction-directed mutagenesis or by using the Quik Change mutagenesis kit (Stratagene). Deletion mutants Sox10DA1–60 and Sox10DA1–102 were generated only in the context of the rat Sox10 cDNA. Plasmid pCMV/T7WS029 was generated by fusing translation initiation consensus and T7 Tag (Novagen) onto the first translation initiation consensus and T7 Tag (Novagen) onto the first residue of the WS029 mutant. The pCMV5-based plasmid pCMV/POU contained the POU domain of Tst-1/Oct6/SCIP as a MunI/KpnI fragment. This plasmid was fused by the calcium phosphate technique as described (11). COS cells were co-transfected with 10% fetal calf serum and transfected by the DEAE-dextran technique as described (11). COS cells were co-transfected with 10% fetal calf serum and transfected by the DEAE-dextran technique as described (11). COS cells were co-transfected with 10% fetal calf serum and transfected by the DEAE-dextran technique as described (11). COS cells were co-transfected with 10% fetal calf serum and transfected by the DEAE-dextran technique as described (11). COS cells were co-transfected with 10% fetal calf serum and transfected by the DEAE-dextran technique as described (11). COS cells were co-transfected with 10% fetal calf serum and transfected by the DEAE-dextran technique as described (11).
Western blots and gel retardation assays (6, 13). TheSX oligonucleotide, which contained a consensus recognition site for Sox proteins (6), was used as probe in electrophoretic mobility shift assays. 1:3000 dilutions of rabbit antiserum against Sox10 (6) or the POU domain of Tst-1/Oct6/SCIP, and a mouse monoclonal against the T7 tag (Novagen), served as primary antibodies in Western blots. 

RESULTS

The four mutations that we previously characterized in one Sox10 allele of Shah-Waardenburg patients are schematically summarized in Fig. 1A. Mutant WS029 is characterized by a nonsense mutation, which converts tyrosine 83 to a stop codon. In the MIC nonsense mutant, glutamate at position 189 is targeted so that the truncated protein consists only of the 188 amino-terminal residues. Mutant 059 lacks the last 106 amino acids of Sox10 due to a deletion of two nucleotides at position 1076 and a resulting frameshift. Mutant 095, on the other hand, carries an insertion of 6 nucleotides between positions 482 and 483. The resulting addition of a leucine and an arginine residue into the DNA-binding HMG box leaves the open reading frame intact.

The mutations were introduced into both rat and human cDNAs, and the resulting mutant proteins were expressed in transiently transfected COS cells. To verify expression, we performed Western blot analysis on whole cell extracts of transfected cells using a polyclonal rabbit antiserum against Sox10. As evident from Fig. 1B, proteins of the expected size were readily detected for mutants 095, 059, and MIC in both human and rat cDNA contexts. Mutant WS029 was not detected due to the inability of our antiserum to detect the first 82 amino acids of Sox10 (data not shown). However, we were able to detect WS029 in a Western blot with a monoclonal T7 tag antibody after amino-terminal addition of the corresponding epitope (see Fig. 7). Tagged and untagged version of WS029 behaved identical in all functional assays.

We have previously failed to detect inherent transcriptional activity when testing the rat Sox10 protein for its ability to stimulate a promoter consisting of a TATA box and multiple Sox consensus recognition sites (6). To extend this analysis, we now carried out similar transient transfection experiments with the human Sox10 protein. As a negative control we included rat Sox10. As a positive control, we used Sox11 from rat, which had previously been shown to be a strong activator of this promoter (14). In the experiments summarized in Fig. 2A, Sox11 stimulated promoter activity approximately 25-fold. Human Sox10 exhibited an intermediate behavior, yielding on average, a 7.5-fold reporter gene induction (Fig. 2A). Thus, the human and rat Sox10 proteins differ from each other with respect to their transactivation capacity. Whereas the presence of rat Sox10 barely influenced reporter gene expression, human Sox10 led to a reproducible reporter gene induction.

This allowed us to test the effect of each mutation on the ability of the human Sox10 protein to activate transcription. As shown in Fig. 2B, all mutations nullified transactivation ability of human Sox10. This result impressively supports our assumption that all of the previously detected human Sox10 mutations lead to functional inactivation of the protein. This result is especially intriguing with respect to mutant 059, which only lacks the last 106 amino acids. Similar experiments with mutations being introduced into the rat cDNA were not informative because of the inability of the wild-type rat Sox10 protein to transactivate in this assay (data not shown).

From the behavior of the 059 mutant, it can be concluded that the carboxyl-terminal region of human Sox10 is required for transcriptional activation. To analyze whether this region would also be sufficient for transcriptional activation, we fused amino acids 354–466 of human Sox10 to the POU domain of Tst-1/Oct6/SCIP (Fig. 3A), thus allowing us to compare the activity of this chimera with the activities of full-length Tst-1/Oct6/SCIP and the isolated POU domain in transient transfections. All constructs were expressed at comparable levels and bound to a recognition site for POU proteins (Fig. 3, B and C). Full-length Tst-1/Oct6/SCIP activated a POU-responsive promoter approximately 5.5-fold in this set of experiments (Fig. 3D). The isolated POU domain was inactive as shown previously (13). Using the Sox10/POU domain chimera, we on average obtained a more than 10-fold stimulation of the luciferase reporter, indicating that the last 113 amino acids of human Sox10 indeed contain a transactivation domain.

Previous analyses had shown that rat Sox10, though not a transcriptional activator by itself, exhibited the ability to synergistically enhance the activity of other transcription factors on suitable promoter constructs (6). Therefore, we tested how the Sox10 mutations would affect this modulatory activity, when introduced into the context of the rat sequence. As shown previously, wild-type rat Sox10 enhanced the transcriptional activity of Tst-1/Oct6/SCIP significantly, both at low and at saturating promoter levels (Fig. 4A and data not shown). Mutants WS029 and 095 both failed to synergistically stimulate the activity of Tst-1/Oct6/SCIP. This was not so with the other two Sox10 mutations. MIC and 059 were approximately as efficient as wild-type rat Sox10 in their ability to enhance Tst-1/Oct6/SCIP function.

Similar experiments were also carried out with human Sox10 and the four mutants introduced into the human cDNA. Again, WS029 and 095 failed to exhibit any synergistic activity.
In the context of the human sequence, mutants 059 and MIC also showed a marked reduction of their ability to enhance Tst-1/Oct6/SCIP function. Whereas the presence of wild-type human Sox10 increased Tst-1/Oct6/SCIP-dependent reporter gene induction from a mere 3-fold to more than 30-fold, 059 and MIC only led to a 10–12-fold promoter stimulation. These induction rates were still significantly higher than the sum of the induction rates obtained for the reporter with either transcription factor alone. Thus, part of the ability to synergistically enhance Tst-1/Oct6/SCIP function is retained in those two mutants. On the basis of these analyses, it can be concluded that the carboxyl terminus, which is missing in MIC and 059, is not absolutely required for synergistic function.

We had previously described a mutant version of rat Sox10 in which most amino acids in front of the HMG domain had been deleted (6). Contrary to the 059 and MIC mutations described here, this ΔN mutant had failed to functionally interact with Tst-1/Oct6/SCIP, indicating that the “synergy domain” might be present in the region amino-terminal of the HMG domain. To study this domain in more detail, we generated two additional mutant versions of rat Sox10 in which we deleted parts of the amino-terminal region. Mutant Sox10Δ1–60 was devoid of the first 60 amino acids, which among Sox proteins are unique to Sox10 (Fig. 5A). Mutant Sox10Δ61–102 on the other hand carried a deletion encompassing amino acids 61–102, which are conserved between Sox10 and the related Sox9 and SoxP1 proteins. Both mutant proteins were effectively expressed in transiently transfected cells and correctly localized to the nucleus as evident from Western blot analysis of nuclear extracts (Fig. 5B). However, when tested for their ability to functionally interact with Tst-1/Oct6/SCIP, only Sox10Δ61–102 proved active, whereas Sox10Δ1–60 was unable to substitute for the wild-type rat Sox10 protein. Thus, we conclude that amino acids 1–60 are involved in mediating synergy with Tst-1/Oct6/SCIP.

To further characterize the impact of the naturally occurring Sox10 mutations, we next tested the ability of each of the mutant proteins to bind to a consensus DNA recognition element for Sox proteins in gel retardation assays (15). Wild-type Sox10 and the 059 and MIC mutants each yielded protein-DNA complexes of characteristic mobility when analyzed in the context of the rat proteins (Fig. 6A). Levels of each Sox10 protein were adjusted to each other by Western blot analysis so that approximately equal amounts of each protein were used in gel retardation assays (Fig. 6B). Nevertheless, the MIC mutant was reproducibly more effective in complex formation, indicating that the truncated protein might have a higher affinity for the Sox consensus recognition site than wild-type Sox10. In contrast to MIC and 059, WS029 and 095 failed to form protein-DNA complexes. For WS029, this was expected as the protein completely lacks the DNA-binding domain. 059, however, contains an HMG box, which is only altered by insertion of two amino acids compared with wild-type Sox10. Identical results were obtained with the mutant human Sox10 proteins (data not shown). From our gel-shift analyses, it can therefore be concluded that the insertion abolished DNA binding completely.

The HMG box of Sox proteins has also been shown to contain two independent nuclear localization signals (16, 17). These signals are present in the 095, 059, and MIC mutants. In agreement, these mutants preferentially localize to the cell nucleus and are found in nuclear extracts of transfected cells. The nuclear localization signals are, however, missing from mutant WS029. Therefore, we determined its localization within the cell (Fig. 7). As already mentioned, WS029 was not detected by our Sox10-specific antiserum and therefore had to be tagged by a T7 epitope. This epitope does not influence the cellular localization of proteins by itself (18). The same epitope was also added onto the HMG box of Sox10. When the cellular localization of both proteins was analyzed by subfractionation studies, it became evident that WS029 preferentially localized to the cytosol, whereas the epitope-tagged HMG box localized to the nucleus (Fig. 7). Thus, we conclude, that WS029 does not reach the nuclear compartment and that the Sox10 HMG box indeed contains the signals necessary for nuclear localization.

**DISCUSSION**

Members of the Sox family of transcriptional regulators are expressed at various stages and in various tissues of the developing embryo (1). Some members have been shown to play important roles in segmentation, development of the nervous system, hematopoiesis, chondrogenesis, and sex determination (3, 19–23). Their inactivation can lead to several diseases in humans (24–27).

We have recently identified Sox10 as a Sox gene preferentially expressed in neural crest cells during early development and later in glial cells of both the peripheral and central nervous system (6). Inactivation of the Sox10 gene was also found to lead to a combination of neural crest defects in the spontaneous
FIG. 4. Synergistic activities of Sox10 mutants. The luciferase reporter plasmid 3×FXO luc, which carries binding sites for both Sox and POU proteins, was transfected into U138 glioblastoma cells in combination with empty CMV expression plasmid (−), expression plasmids for Tst-1/Oct6/SCIP, wild-type Sox10, Sox10 mutants WS029, 095, MIC, and 059, or various combinations thereof, as indicated. Luciferase activities were determined in three independent experiments, each performed in duplicate. Values from transfections with luciferase reporter and empty expression plasmid were arbitrarily set to 1. Data for all other transfections are presented as -fold induction above this level. A, mutations introduced into the rat Sox10 sequence; B, mutations introduced into the human Sox10 sequence.

FIG. 5. Sox10 domains involved in synergism with Tst-1/Oct6/SCIP. A, summary of Sox10 mutants. Sox10Δ1–60, mutant rat Sox10 lacking amino acids 1–60; Sox10Δ61–102, mutant rat Sox10 lacking amino acids 61–102. B, comparison of expression levels between Sox10 and its mutants Sox10Δ1–60 and Sox10Δ61–102 in nuclear extracts of transfected cells by Western blot using rabbit antiserum against Sox10. Numbers on left indicate size of molecular mass markers in kilodaltons. C, the luciferase reporter plasmid 3×FXO luc was transfected into U138 glioblastoma cells in combination with empty CMV expression plasmid (−) or expression plasmids for Tst-1/Oct6/SCIP (Tst-1), wild-type Sox10, or the Sox10 mutants Δ1–60 and Δ61–102 as indicated. Luciferase activities were determined in three independent experiments, each performed in duplicate. Values from transfections with luciferase reporter and empty expression plasmid were arbitrarily set to 1. Data for all other transfections are presented as -fold induction above this level.

mouse mutant Dominant megacolon (7, 8). Furthermore, mutations in the Sox10 gene were shown to underlie some cases of combined Waardenburg-Hirschsprung disease in humans (9). In this disease, the aganglionic colon characteristic of Hirschsprung disease is combined with sensorineural deafness and pigmentation abnormalities as usually seen in Waardenburg syndrome.

Four independent Sox10 mutations were identified so far in human patients, three with a sporadic and one with a familial occurrence (9). Here, we have characterized the impact of each of these mutations on Sox10 function in vitro. Our results indicate that all mutations interfere with normal Sox10 function. Mutant WS029, which corresponded to the first 82 amino acids of Sox10, exhibited an aberrant cytosolic localization and failed to bind to DNA. Previous analyses on Sox9 and Sry, the prototypic member of the Sox gene family, had led to the detection of two independent nuclear localization signals within the amino-terminal and the carboxyl-terminal end of the HMG domain (16, 17). These signals are conserved in most Sox proteins, including Sox10. The occurrence of nuclear localization signals within the HMG domain of Sox10 is also corroborated by the nuclear localization observed in this study for the isolated HMG domain. Thus it seems that both the aberrant cellular localization and the failure to bind to DNA can be attributed to the loss of the HMG box in WS029. Given the severity of the mutation, it is not surprising that WS029 failed to show intrinsic transcriptional activation as well as synergistic activity with Tst-1/Oct6/SCIP.

Mutant 095 showed the typical nuclear localization but failed to bind to DNA. This failure was due to the insertion of 2 amino acids into the DNA-binding HMG box. The insertion affects a region that is folded into helix 3, one of the three α-helices, which together form the twisted L-shaped structure.
of the HMG box (4). Additionally, helix 3 is normally involved in direct contacts with DNA. The 095 insertion in helix 3 therefore either interferes with DNA binding directly or alters the overall conformation of the HMG box such that DNA binding is disturbed. Similar to WS029, 095 exhibited no transcriptional activity, either alone or in combination with other transcription factors such as Tst-1/Oct6/SCIP.

The other two mutants, MIC and 059, differed from WS029 and 095 in that they showed no obvious defects in cellular localization or DNA-binding. In agreement with this, both mutants contained an intact HMG box. When tested in the context of the rat sequence for their ability to synergistically enhance the activity of Tst-1/Oct6/SCIP, these mutants behaved very similar to wild-type Sox10 protein from rat. This implied that the region required for synergistic enhancement was present in both MIC and 059. As MIC only consists of the first 188 amino acids of Sox10, the synergy domain should be present within this region. In agreement, we could show in this study that amino acids 1–60 are required for synergistic activation.

Given the fact that the frameshift previously identified in the Dom mouse (7, 8) occurs at a position very close to the nonsense mutation found in MIC, it is of particular interest to compare the effect of both mutations. Both mutants contain almost the exact same Sox10 sequences, which in case of the Dom mutant are followed by 99 amino acids normally not found in Sox10. Whereas introduction of the MIC mutation in the rat cDNA leaves synergistic function intact, as shown in this paper, introduction of the Dom mutation abolished synergistic activity of the resulting mutant Dom protein altogether (7). This divergent behavior of both mutant proteins points to an inhibitory role for the Dom-specific carboxyl-terminal domain. It is our hypothesis that the function of the amino-terminal synergy domain is sensitive to intramolecular regulation by other parts of the protein and that the Dom-specific carboxyl terminus represses it.

Whereas no defects could be detected for the MIC and 059 mutations in the context of the rat sequence, functional deficits were obvious for both of these mutations in the context of the human sequence. This was largely due to the fact that the wild-type human Sox10 protein exhibited a significant autonomous transcriptional capacity previously undetectable in the rat protein. At present it is not clear what causes this differential behavior of the rat and the human protein. Given the fact that both proteins only differ by 12 amino acids, it will be interesting to analyze which of these amino acid differences causes the altered behavior. This species-specific difference is not without precedence among Sox proteins, as only the murine, but not the human Sry protein functions as a transcriptional activator (28).

Even the human Sox10 protein, however, exhibited only a modest transactivation capacity, which is far below the activity observed with other transcription factors in this kind of assay. Although some Sox proteins show a strong transactivation capacity (29–32), most family members are only weak transcriptional activators at best (6, 33–37). This has led to the assumption that Sox proteins primarily function as accessory proteins in combination with other transcription factors. Alternatively, Sox proteins might be active in heteromeric protein complexes in which a separate transactivation domain is provided by other proteins within the complex. Such a mechanism has been recently established for the related HMG box proteins of the Lef/Tcf family (38, 39). Given their ability to introduce large conformational changes into DNA, it has been suggested that HMG box-containing proteins perform their accessory role as architectural proteins, which arrange such DNA-bound multiprotein complexes as the enhanceosome (5, 40).

Notwithstanding the exact mechanism of Sox10 function, the inherent transcriptional activity of human Sox10 allowed us to test each of the four mutations found in human patients for their ability to transactivate. Our results clearly show that all mutations, including 059 and MIC, severely impaired the transcriptional activity of Sox10. 059 contains all Sox10 sequences except for the last 106 amino acids and still failed to show transcriptional activity. In a complementary experiment, fusion of the last 112 amino acids of human Sox10 to the POU domain of Tst-1/Oct6/SCIP led to the reconstitution of an effective transcriptional activator, thus arguing that the carboxyl-terminal region of human Sox10 is both necessary and sufficient for transactivation. In those Sox proteins where it has been analyzed, transactivation domains have often been found in the carboxyl-terminal part of the protein (14, 28–32, 36).

Interestingly, the transactivation domain of the structurally related Sox9 protein has also been mapped to the extreme carboxyl terminus (30, 31). Sox9 is not only structurally related to Sox10, but like Sox10 causes a disease in humans (25, 27). This disease is known as campomelic dysplasia, a rare, often lethal, dominantly inherited, congenital osteochondrodysplasia, associated with male-to-female autosomal sex reversal in two-thirds of the affected karyotypic males. Mutations in the affected Sox9 allele are frequently found within or in close proximity to the DNA-binding domain or lead to a loss of the carboxyl-terminal transactivation domain (31, 41) similar to the mutations described for Sox10.

Sox9 and Sox10 exhibit an amino acid identity of 59% over the 95 carboxyl-terminal residues. Thus it is conceivable that these amino acids are involved in the transactivation function. Given the substantial differences in the transactivation capacities between Sox9, rat, and human Sox10, however, it is unlikely that these conserved amino acids are sufficient for transactivation function. The nonconserved amino acids very likely participate by modulating the strength of the transactivation domain.

Also, it is interesting to note that in the human Sox10 protein the transactivation domain communicates with the synergy domain on a functional level. Thus synergy rates are higher for the human protein than for the rat protein. Deletion of the transactivation domain from the human protein as observed in MIC and 059 led to a substantial reduction in the observed synergy by reducing it to the level of the rat protein. Thus, the bona fide carboxyl terminus of human Sox10 has exactly the opposite effect on the function of the synergy domain as the Dom-specific carboxyl terminus.

From our in vitro analyses, we conclude that the functional defects are more severe in the WS029 and 095 mutants than in the 059 and MIC mutants. Given the strong influence of the genetic background in both Hirschspring disease and Waardenburg syndrome (10, 42), it is not easy to predict the severity of the disease from the behavior of a certain mutation in the in vitro tests. However, we like to point out that 059, the mutant in which only the last 106 amino acids are missing, is different from the other mutants in two respects. First, 059 is the only familial mutation; second, 059 only led to a combination of Waardenburg-Hirschspring symptoms in some family members affected by this mutation, whereas others suffer from a mild form of Waardenburg syndrome. Thus, 059 seems to be a relatively weak mutation, arguing that there might exist a limited correlation between the behavior of a mutation in vitro and its effects in vivo.

Acknowledgment—We thank Janna Enderich for expert technical assistance.
