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Original Article

In vitro binding and expression studies demonstrate a role for the mouse Sry Q-rich domain in sex determination

JIANQING ZHANG, PETER COWARD, MEIWEI XIAN and YUN-FAI C. LAU*

Division of Cell and Developmental Genetics, Department of Medicine, University of California, San Francisco, USA

ABSTRACT The Q-rich domain of the mouse sex determining gene, *Sry*, is encoded by an in-frame insertion of a repetitive sequence composed of mostly CAG repeats. The exact function of this Q-rich domain is unknown. Studies on the polymorphisms within this Q-rich domain among different *domesticus* and *musculus* mouse strains suggest a possible role for this domain in sex determination. Using the farwestern protein-blotting technique and recombinant fusion proteins containing the *Sry* Q-rich domain as probes, three Sry interactive proteins of 94, 32 and 28 kDa apparent molecular weight (Sip-1, -2 and -3 respectively) were consistently detected in adult testis. Sip expression was detected in somatic cells and was associated with the spermatogenic activity of the testis. During embryogenesis, Sips were readily detected in total tissue extracts of embryos as early as E8.5 day. In fetal gonads of both sexes, their expression peaked around E11.5-13.5 day, at the time of sex determination and differentiation, and decreased drastically towards late stages of gestation. These observations support the hypothesis that the Q-rich domain may contribute to the biological function(s) of mouse Sry through a protein-protein interactive role(s).

KEY WORDS: *Sry, interactive proteins, CAG repeats*

Introduction

The mouse sex determining gene, Sry, on the Y chromosome encodes a protein that harbors two major domains: the amino terminal DNA-binding domain and the carboxyl terminal glutamine-rich (Q-rich) domain (Koopman *et al*., 1991; Gubbay *et al*., 1992; Goodfellow and Lovell-Badge, 1993; Coward *et al*., 1994; Greenfield and Koopman, 1996). The DNA-binding domain contains a high mobility group motif (HMG box) and is evolutionarily conserved among the *Sry* genes of all mammalian species analyzed so far. The HMG box binds to the target sequence A/TACAAT (Harley *et al*., 1994) and bends the DNA about 80° *in vitro* (Ferrari *et al*., 1992; Harley *et al*., 1994; Pontiggia *et al*., 1994, 1995). Mutations in the HMG box have been associated with sex reversal in man (Harley *et al*., 1992; Hawkins *et al*., 1992). The Q-rich domain is extremely unusual since it is present only in the mouse Sry, but not those of other mammals (Foster *et al*., 1992; Su and Lau, 1993; Tucker and Lundrigan, 1993; Whitfield *et al*., 1993; Daneau *et al*., 1995, 1996). It is encoded primarily by an in-frame insertion of a repetitive sequence composed of mostly CAG repeats. The exact function of this Q-rich domain is unknown. Studies of *Sry* gene among different *domesticus* and *musculus* strains have demonstrated several polymorphisms within the CAG repeats that can be correlated to the B6.Y^{Dom} sex reversal, a condition which occurs when the Y chromosome from certain *domesticus* strains is introduced to the genetic background of the inbred strain, C57BL/6J (B6) (Coward *et al*., 1994). We hypothesize that this Q-rich domain is a protein-binding motif and contributes to the function of Sry by interacting with a co-factor(s) involved in the sex determination process. In this model, the polymorphism between *musculus* and *domesticus* Sry has led to slightly different mode(s) of interaction between the respective *Sry* Q-rich domain and the co-factor(s), resulting in various degrees of sex reversal in B6.Y^{Dom} animals.

To test this hypothesis, we have performed farwestern blotting analysis of protein extracts derived from both adult and fetal tissues using protein probes derived from different parts of the mouse Sry. We have demonstrated that three proteins, termed Sry interactive protein-1, -2 and -3 (Sip-1, -2 and -3), of apparent molecular weights of 94, 32 and 28 kDa respectively, bind specifically to the Sry Q-rich domain in the adult testis. The Sips are detected in somatic cells of testicular origins. Their expression is associated with spermatogenic activity in normal testis and is proportional to the amount of testicular tissue in the gonads of adult B6.Y^{Dom} mice. Further, Sips are expressed in tissues of embryos as early as E8.5 day and in fetal gonads of both sexes at E11.5 day, during the time

Abbreviations used in this paper: HMG box, High mobility group box; Q-rich, glutamine-rich; Sry, sex determining region Y; Sip, Sry interactive protein; B6.Y^{Dom}, domesticus Y chromosome in C57BL/6 background; *Wt-1*, Wilm's tumor; *Sf-1*, steroidogenic factor 1.

***Address for reprints:** Division of Cell and Developmental Genetics, Department of Medicine, VAMC-111C5, University of California, San Francisco, 4150 Clement Street, San Francisco, CA 94121, USA. FAX: 415 502-1613. e-mail: clau@itsa.ucsf.edu

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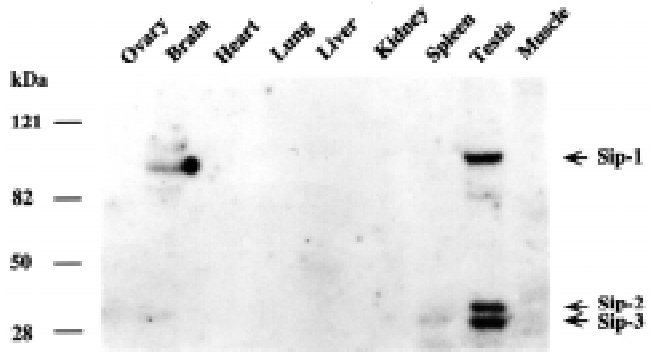

Fig. 1. Detection of novel Sry interactive proteins in the adult mouse testis and brain. *Mouse tissue extracts (100 μg each) derived from female ovaries and male brain, heart, lung, liver, kidney, spleen, testis and muscle were analyzed by farwestern blotting using ^{32}P-labeled GST-B6Sry-Q as a probe. Three specific proteins of 94, 32 and 28 kDa, designated as Sry interactive protein -1, -2 and -3 (Sip-1, -2, -3), were consistently observed in the testis sample. A minor band at 90 kDa was also detected in the brain.*

of sex determination. However, their expression patterns vary both quantitatively and qualitatively at different developmental stages. These findings support the hypothesis that the Q-rich domain serves an important role(s) in the proper functioning of the *Sry* gene through a protein-protein interaction mechanism.

Results

Detection of specific Sry interactive proteins in adult testis

To demonstrate the protein-protein binding activities of the Q-rich domain of the mouse Sry, total protein extracts from various tissues of adult mice were analyzed by farwestern blotting with labeled GST-Sry fusion protein probes (Kaelin *et al.*, 1992, see Fig. 11 for description of constructs). Initial experiments demonstrated that 3 prominent proteins of apparent molecular weight 94, 32 and 28 kDa were consistently detected in the protein extract of the testis, but not those of other tissues, by a GST fusion protein (B6Sry-Q) probe containing the Q-rich domain from the B6 *Sry* gene (Fig. 1). We have designated these testis-specific proteins as the Sry interactive protein-1, -2 and -3 (Sip-1, Sip-2 and Sip-3) for the 94, 32 and 28 kDa testicular proteins respectively. Similar experiments using a fusion protein probe consisting of the HMG box from B6 Sry did not detect any interactive protein.

Since the CAG repeats are only present in the mouse *Sry* gene, the specificity of the interactions between the B6 Sry Q-rich domain and Sip-1, -2, -3 were further determined by farwestern blotting of total protein extracts from testes of human, chimpanzee, dog, rabbit, guinea pig, wood lemming and rat using the B6Sry-Q probe. While the Sips were present in the testis extracts of B6 and *domesticus* (Dom Tirano) mice, no Sip of any forms were detected under the same conditions in the testes of other mammals examined (Fig. 2). The detection of all the Sip proteins in the B6 and Dom testis extracts indicate that Sips from both strains interact with the B6Sry-Q probe with similar efficiency.

Competition assays were performed to further demonstrate the specificity of the interactions between the Sry-Q domain and the Sips. Binding was measured using identical SDS-PAGE strips of testis extracts from B6 and *domesticus* (Tirano) in farwestern blotting analyses. Labeled GST alone did not show any significant binding to the testis extracts (Fig. 3A, left 2 lanes). The Sry-Q probe from B6 strain consistently detected all 3 Sips in both B6 and *domesticus* (Tirano) testis extracts (Fig. 3A, right two lanes). However, when unlabeled Sry-Q recombinant proteins from either the B6 or *domesticus* (Tirano) were co-incubated with the B6Sry-Q probe in the hybridization mixtures, the binding of the B6Sry-Q probe to the respective Sips were drastically suppressed at 10X excess and were abolished at 50X and 250X excess of the respective competitors (Fig. 3B,C). Co-incubation of excess recombinant protein containing the HMG box domain did not compete with the B6Sry-Q probe for Sip bindings (Fig. 3D). These results demonstrate highly specific binding between the Sry Q-rich domain and the respective Sips.

Sips are present in both musculus and domesticus testes

Our previous studies demonstrated that a CAG polymorphism at the Q-rich domain of the *Sry* gene correlates with B6.Y^{Dom} sex reversal (Coward *et al.*, 1994). The B6 and 129 strains are *musculus* strains harboring a *molossinus* Y chromosome (Coward *et al.*, 1994; Hacker *et al.*, 1995; Miller *et al.*, 1995). Their *Sry* genes contain 12 CAG repeats at the polymorphic site, at codon 170-181 inclusively. The *domesticus* strains, FVB/N and SJL/J, contain the same number of CAG repeats at the same site of their *Sry* genes, their Y chromosomes do not produce any sex-reversal in B6 background. The *domesticus* strains, AKR/J and PL/J, have 13 CAG repeats in the same site, the sex reversal effect of their Y chromosome in B6 background is manifested as a delay in testis development during embryogenesis. The *domesticus* strains from Tirano and Zalende *(m. poschiavinus)* have 11 CAG repeats at the polymorphic site of their *Sry* genes, their Y chromosomes produce an adult sex-reversal effect in a B6 background (Eicher *et al.*, 1982; Eicher and Washburn, 1983; Nagamine *et al.*, 1987; Biddle and Nishioka, 1988; Coward *et al.*, 1994). Although we cannot rule out the possibility that the CAG polymorphisms may be caused by founder effects and may not be responsible for the B6.Y^{Dom} sex reversal (Carlisle *et al.*, 1996), the results show that the Sry-Q domain indeed interacts with specific

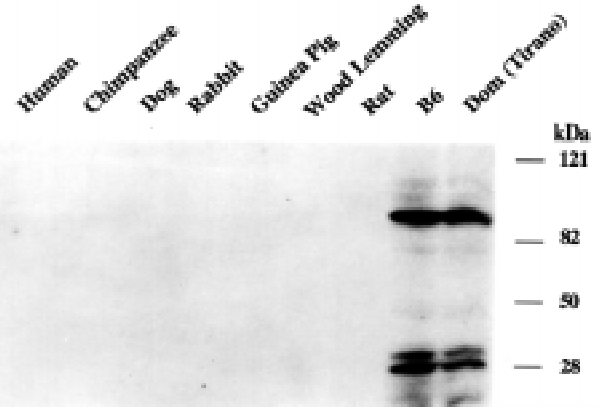

Fig. 2. Sips are undetectable in testes of other mammals. *Farwestern blotting was used to examine the expression of Sips in total protein extracts from testes of human, chimpanzee, dog, rabbit, guinea pig, wood lemming, rat and B6 and Dom(Tirano) mice. The blots were probed with a ^{32}P-labeled GST-B6Sry-Q. The Sips are readily detected only in the mouse testes.*

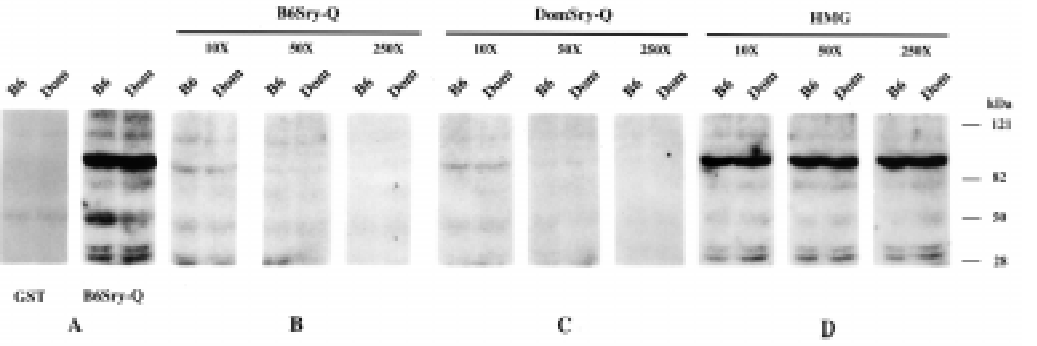

Fig. 3. The Sry-Sip interactions are specific for the Q-rich domain of Sry. *As a control, GST protein was labeled and used in farwestern blotting of B6 and Dom testis extracts. No specific interactions were observed* (A, *GST, left 2 lanes). The same experiment was performed with a* ^{32}*P-labeled GST-B6Sry-Q probe (containing the Q-rich domain of B6 Sry), the specific Sips in both mouse testis samples were detected (A, B6-Sry-Q, right 2 lanes). Competition experiments were performed with unlabeled proteins as competitor at 10X, 50X and 250X excess to the labeled B6Sry-Q probe. The filters containing the mouse testis extracts were co-incubated with cold PAR-B6Sry-Q* **(B)**, *PAR-DomSry-Q* **(C)** *or PAR-HMG* **(D)** *fusion proteins. The Sry-Sip binding was drastically reduced with both B6 and Dom Sry-Q domains as competitors at 10X excess and were abolished at higher competitor to probe ratios (B and C) while little effect was observed with the HMG competitor protein (D). The corresponding gene constructs were described in Figure 11. Plasmids PAR-B6Sry-HMG, PAR-B6Sry-Q and PAR-DomSry-Q were used to generate competitor proteins B6Sry-Q, DomSry-Q and HMG respectively for the present experiment.*

Sips. Hence, the polymorphisms may lead to differential interactions between the Sry-Q and Sips. Therefore, it seems logical to address the following two questions: 1) Are Sips present in the testes of other *musculus* and *domesticus* strains? 2) Are there any differences in the interactions between the B6Sry-Q and the DomSry-Q with Sips?

We performed farwestern blotting on protein extracts of testes from representative mouse strains of the three classes of B6.Y^{Dom} sex reversal (Coward *et al.*, 1994) using either B6Sry-Q or DomSry-Q probes containing the Q-rich domain from the B6 or *domesticus* (Tirano) Sry protein respectively. Results from these experiments indicate that the Sips were detected in all *musculus* and *domesticus* mouse testes, using either the B6Sry-Q or DomSry-Q fusion protein probes (Fig. 4A,B respectively). However, under similar conditions, DomSry-Q bound less intensely to the Sips than B6Sry-Q. Noticeably, the bands ascribed to the binding of DomSry-Q to Sip-2 were barely detectable in most samples (Fig. 4B). Further, an additional band at 48 kDa was also detected by this probe. The differences in the Sry-Sip interactions seemed to be dependent on the Q-rich probe used and not the testis samples in each experiment. These observations, if confirmed by in vivo binding procedures or other means, imply that the polymorphisms between the Q-rich domains of B6 and Dom Sry were responsible for the variable Sry-Sip interactions.

Sips are associated with testicular tissues in B6.Y^{Pos} gonads

A breeding colony of B6.Y^{Pos} mice was established in our laboratory with founding animals *(domesticus poschiavinus)* obtained from the Jackson Laboratory. To correlate their gonadal development and Sip expression, gonads from adult animals were collected and classified as large XY testes, small XY testes, XY ovotestes, XY ovaries and XX ovaries. They were then analyzed by farwestern blotting with B6Sry-Q as a probe (Fig. 5). The results show that the Sips were expressed similarly in both normal B6 testis control (lane 1) and those from large testes (lane 2) in B6.Y^{Pos} animals. The level of Sip-1 was gradually reduced in small testis (lane 3) and ovotestis (lane 4) while those for Sip-2 and -3 remained relatively unchanged. Interestingly, a 120 kDa band was also observed in the testis samples. Its intensity also decreased in parallel with that of Sip-1. In XY ovaries, these two small Sips as well as the larger Sip-1 were also observed. In particular, a 85 kDa band was also detected prominently (lane 5). The Sips were not detected in the XX ovary control (lane 6). With the exception of the XY ovary sample, these results suggest that expression of the Sips is directly proportional to the amount of testicular tissues, and probably spermatogenic activity, within the gonads of these sex-reversed animals. In case of the XY ovary sample, we are not certain whether the Sip signals were derived from pockets of testicular cells within these morphologically ovarian structures or the Sips were aberrantly expressed in XY ovaries.

Sips are associated with somatic cells and spermatogenesis of the testis

To determine if interactions between the Sry-Q domain and Sips are associated with spermatogenesis in adult testis, postnatal testes were prepared from mice at 0.5 (newborn), 6, 12, 18, 21, 42 and 112 days after birth and analyzed by farwestern blotting with the GST-B6Sry-Q probe. Results from this experiment indicate that Sips were present at low levels in the testis as early as newborn. Their abundance gradually increased as the testis ma-

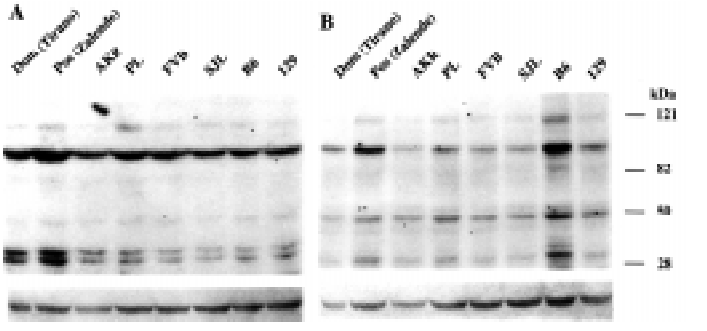

Fig. 4. Detection of Sips in testes of both *musculus* and *domesticus* strains. *Testis extracts from domesticus strains, Dom (Tirano), poschiavinus (Zalende), AKR, PL, FVB, SJL, and musculus strains, B6 and 129 were size-fractionated by SDS-PAGE and probed with GST-B6Sry-Q* **(A)** *and GST-DomSry-Q* **(B)**. *Although the Sips were detected in the protein extracts from the testes of all mouse strains by both Sry-Q domain probes, the DomSry-Q probe bound less intensely to the Sips, particularly to Sip-2 (B), than the B6Sry-Q probe (A). A 48-kDa band was also observed in the blot with DomSry-Q probe. Western blotting of the same filters with anti-tubulin antibody showed approximately equal loading of respective testis extract for each lane (A and B, lower panels).*

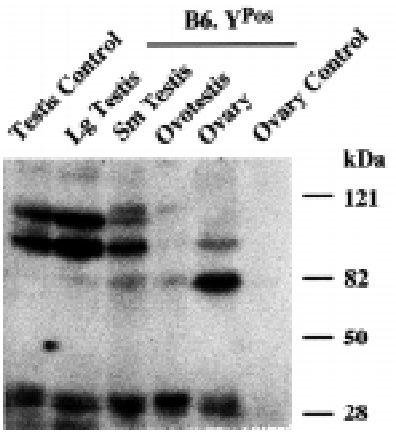

Fig. 5. Expression of Sips in B6.Y^{Pos} gonads. *Gonads from B6.Y^{Pos} mice were dissected and pooled according to the following morphology categories: large testis (lane 2), small testis (lane 3), ovotestis (lane 4) and ovary (lane 5), and analyzed with farwestern blotting using B6Sry-Q as a probe. Adult testis (lane 1) and ovary (lane 6) were used as positive and negative control respectively. The 3 Sips were detected in all B6.Y^{Pos} gonads, except the XY ovary sample that showed an additional prominent 85 kDa variant band.*

tured at 21 and 42 days of age when the spermatogenic process was fully functional (Fig. 6), suggesting that the expression of Sips was associated with spermatogenesis in adult testis. Since the number of germ cells, particularly those at late spermatogenic stages, increases exponentially during the maturation of the mouse testis (Bellve *et al.*, 1977), these findings suggest that the Sips may be expressed in the germ cells. To address the question on whether the somatic cells may be responsible for such increase in Sip synthesis in adult testis, farwestern blotting analysis was performed on two cell lines, TM3 and TM4, which were initially

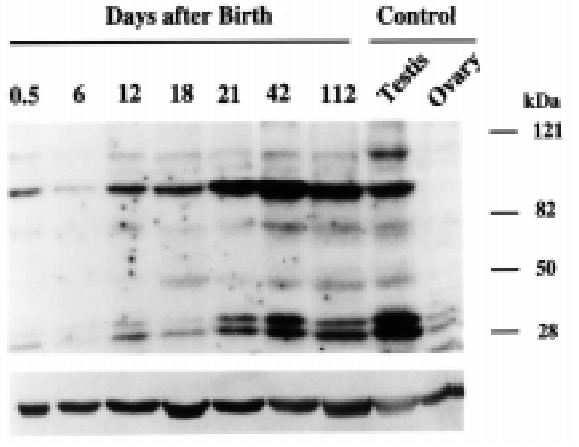

Fig. 6. Sips are associated with spermatogenic activity in the adult testis. *Protein extracts from testes of mice at 0.5 (newborn), 6, 12, 18, 21, 42, and 112 days after birth and control adult testis and ovary were analyzed with farwestern blotting using GST-B6Sry-Q as a probe (upper panel). The Sips were present in all testes, but in relatively high quantity only in testes of mice 21 days of age or older when a full complement of germ cells was present. As a control for protein loading, western blotting was performed on the same filter with an anti-tyrosinated α-tubulin monoclonal antibody (lower panel).*

derived from the Leydig and Sertoli cells of the mouse testis respectively (Mather, 1980). The results demonstrated that the same Sips were detected in both cell lines (Fig. 7A), suggesting that the somatic cells derived from the mouse testis also express Sips. To detect the subcellular locations of the Sips in somatic cells, TM4 cells were fractionated into crude cytoplasmic and nuclear fractions that were analyzed with farwestern blotting. The results demonstrated that Sip-2 and Sip-3 were predominantly present in the nuclear fraction while the 94-kDa Sip-1 was distributed in both nuclear and cytoplasmic fractions (Fig. 7B). Western blotting of the same filter with a monoclonal antibody against a tyrosinated α-tubulin peptide (Kreis, 1987) indicated that α-tubulin was present only in the cytoplasmic fraction and was absent in the nuclear fraction. The above studies, taken together, indicate that the Sips are expressed as mostly nuclear proteins in somatic cells and their expression is associated with the spermatogenic activity of the adult testis.

Localization of Sips in adult testis

The location(s) of Sips in the adult testis was analyzed further by a combined *in situ* protein transfer (Okabe *et al.*, 1993) and

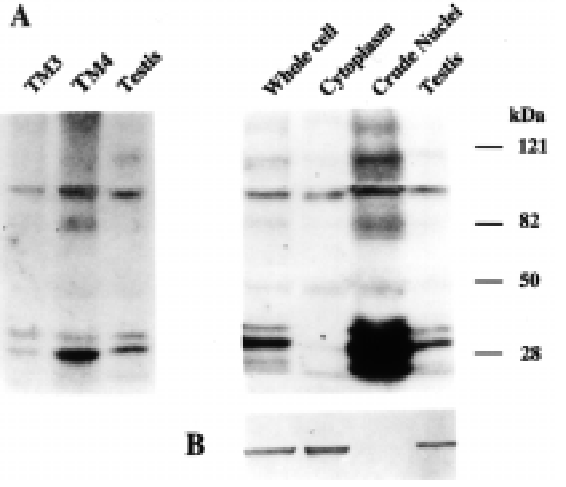

Fig. 7. Subcellular localization of Sips in testicular somatic cells. (A) *Protein extracts of somatic cell lines TM3 and TM4 derived respectively from Leydig and Sertoli cells of the mouse testis were probed with GST-B6Sry-Q. All Sips were detected in both TM3 (lane 1), TM4 (lane 2) and the adult testis control (lane 3).* **(B)** *TM4 cells (lane 1) were sub-fractionated into cytoplasm (lane 2) and crude nuclei (lane 3) and analyzed, together with a testis control (lane 4), by farwestern blotting using GST-B6Sry-Q as a probe. The small molecular weight Sip-2 and -3 were predominantly localized in the nuclei while the larger Sip-1 was distributed in both nuclear and cytoplasmic fractions. Western blotting was performed with the anti-tubulin monoclonal antibody (lower panel). Lanes 1, 2 and 4 were positive and lane 3 was negative for this cytoplasmic protein marker, suggesting that both the cytoplasmic and nuclear fractions were relatively pure.*

farwestern blotting technique. In this approach, an imprint of the proteins from the mouse testis section was transferred *in situ* to a PVDF membrane and was then processed for the farwestern blotting procedure, as described above. ^{35}S-labeled Sry proteins, synthesized by *in vitro* transcription and translation reactions, were used as probes. The Sry-Sip bindings were detected by autoradiography with β Max Hyperfilm (Amersham Inc.). After

autoradiography, the morphology of the tissue imprints was visualized by immunostaining using specific antibodies.

As a preliminary experiment, testis imprints were analyzed by immunohistochemical staining using a specific monoclonal antibody against the tryosinated α-tubulin which is preferentially expressed in somatic cells (Kreis, 1987). Results demonstrated that the *in situ* blotting procedure faithfully transferred proteins directly from the frozen testis section to PVDF membrane (Fig. 8A,B). ^{35}S-labeled protein probes consisting of either the entire B6 Sry molecule (including the HMG box and Q-rich domain) or Q-rich domain were used in farwestern blotting of mouse testis imprints on PVDF membrane. Identical results were obtained in both types of *in situ* farwestern blotting experiments using probes derived from either the entire Sry protein or Q-rich domain alone. Signals were detected in both the interstitial and peripheral regions of the seminiferous tubules where somatic cells were mostly located (Fig. 8C,E). Such locations were subsequently identified by immunostaining of the same imprints with antibodies against either the somatic cell specific tryosinated α-tubulin or germ cell specific Ldh-X (Jen *et al.*, 1990) (Fig. 8D,F). A control experiment using probe derived from the vector alone did not show any significant binding to the testis imprints (data not shown). These findings, therefore, confirm the expression of Sips in the somatic cells of the adult testis and demonstrate that the HMG box did not influence the bindings of the Q-rich domain to the Sips.

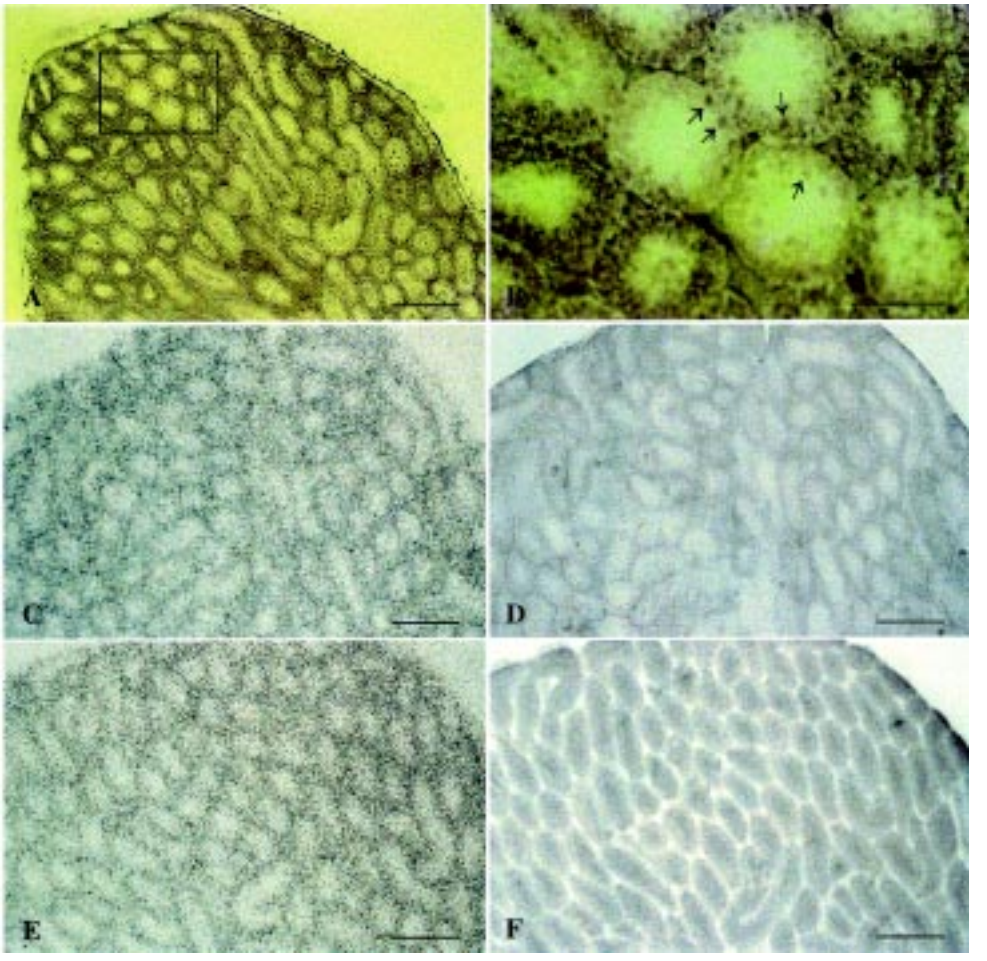

Fig. 8. Localization of Sip proteins by *in situ* farwestern blotting. *Adult mouse testis imprints on PVDF membrane were immunostained with a monoclonal antibody against the α-tubulin. Specific signals were observed in the interstitial cells and the basal lamina region of the seminiferous tubules* **(A)**. *An enlargement of a subsection (boxed) in A showed staining of putative Sertoli cells (arrows) within the tubules* **(B)**. *Farwestern blotting of the testis imprints using the entire Sry protein as a probe indicated that the Sips are primarily located in the somatic cell regions of the testis* **(C,E)**, *as demonstrated by subsequent immunostaining of the corresponding imprints with antibodies against either α-tubulin* **(D)** *or the germ cell*

The Sips are expressed differentially in fetal mouse tissues

If the Sips play any role(s) in sex determination, they must be expressed together with Sry at the time of sex determination. To address this question, we have performed detailed expression studies of the Sips during mouse embryogenesis. Gonads were dissected from fetal mice at E11.5 to E17.5 day and newborn mice and processed for farwestern blotting using B6Sry-Q as a probe. Several interesting results were observed (Fig. 9). First, the Sips were detected in all fetal and newborn gonads of both sexes. Second, their expression was at the highest levels from E11.5 to E13.5 day and gradually decreased toward later stages of embryogenesis. Third, additional bands adjacent to the lower molecular Sips were also observed (e.g., Fig. 9, arrow). The exact nature of these additional bands is unclear. They could potentially represent isoforms of Sip-2 or Sip-3. Fourth, although the Sips were detected in gonads of both sexes at birth, they persisted only in the testis in adults.

To determine if Sips are also expressed in other fetal tissues, the remaining embryos after gonad dissection were pooled according to their developmental stages and sexes and processed similarly for farwestern blotting (Fig. 10A and B for male and female embryos respectively). The Sips expression levels were at the highest from E11.5 to E13.5 day and decreased drastically from E15.5 day to newborn. Noticeably, the 94 kDa Sip-1 band disappeared and a new band of 85 kDa apparent molecular weight appeared in both male and female embryos at E15.5 day. In turn, this 85 kDa band also disappeared in the samples from E17.5 day fetal and newborn mice. Interestingly this 85 kDa band was also observed in XY ovary sample in B6.Y^{Pos} mice. Additional bands (Fig. 10, arrows) were also detected adjacent to Sip-2 and Sip-3, similar to those observed in fetal gonad samples (Fig. 9). The expression levels were relatively higher in the small Sips than the larger Sip-1 in the fetal samples.

Since the expression of the Sips were high in fetal tissues at E11.5 day, we had addressed the question on whether such expression could be detected in embryos at earlier stages of

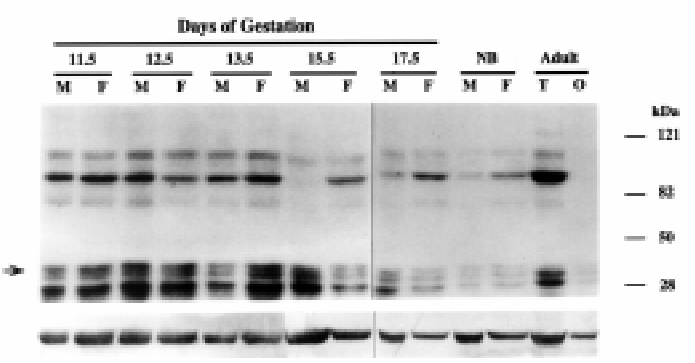

Fig. 9. The Sips are expressed in fetal gonads of both sexes. *Total protein extracts were prepared from fetal gonads from mouse embryos of both sexes (M= male, F= female) at embryonic day 11.5, 12.5, 13.5, 15.5, 17.5 (lanes 1-10), newborn gonads (NB, lanes 11, 12) and adult testis (T, lane 13) and ovary (O, lane 14), and were analyzed by farwestern blotting using GST-B6Sry-Q as a probe. The Sips were detected in all fetal and newborn gonads of both sexes and in adult testis. Their expression levels were the highest at E11.5-E13.5 day among the fetal and newborn samples. The arrow indicates an additional band slightly above the position for Sip-2. Although identity of this extra Sry-Q binding band is uncertain, it can potentially represent an isoform of the smaller Sips. The lower panel represents results from western blotting of the same filters with an anti-tubulin monoclonal antibody.*

development. Farwestern blotting was performed with protein extracts from pooled embryos of both sexes at E8.5 day to E11.5 day. Similarly high levels of Sip expression were observed in all samples at these developmental stages (Fig. 10C). The findings demonstrated that Sips were expressed most abundantly in fetal gonads during the time of sex determination and in tissues of early embryos.

Discussion

The present study has provided supporting evidence for the hypothesis that the Q-rich domain of the mouse Sry plays a role in sex determination by interacting with Sips. However, two groups of investigators had recently reported additional polymorphisms at the CAG repeated region of the *Sry* genes of other *domesticus* and *musculus* strains (Carlisle *et al.*, 1996; Albrecht and Eicher, 1997). Some of these changes do not correlate to the degrees of sex reversal when the respective *domesticus* Y chromosome is introduced into B6 genetic background. Based on DNA sequencing information, Albrecht and Eicher further suggested that only the first 143 amino acids, consisting of the HMG box and adjacent sequence but excluding the Q-rich domain, is sufficient for Sry function. These investigators concluded that misregulation of the *domesticus Sry* gene may be responsible for the sex reversal in B6.$Y^{Pos/Dom}$ mice. This postulation is in contrast to the results of the present study that demonstrate the Q-rich domain to be a protein-protein interactive domain. It interacts with at least 3 specific Sip proteins. Although the Sips are more widely distributed in both male and female gonads and tissues during embryogenesis, their expression patterns are compatible with a role(s) in sex determination. First, the Sips are most abundant at E11.5 to E13.5 day in the fetal gonads, thereby placing these proteins in the gonads at the time when Sry mediates a male sex determination. By virtue of their detection by a protein-binding assay, the Sip proteins should be present in the fetal gonads and be available for interaction with the Q-rich domain of Sry. Second, transgenic mouse studies had demonstrated that introduction of a functional *Sry* gene in XX embryos is sufficient for male sex determination (Koopman *et al.*, 1991), suggesting that other sex-determining genes are expressed in female embryos and are available for the completion of this developmental pathway. The Sips are potential candidates for such sex-determining (co-factor) genes. However, we recognize that our data are obtained by an *in vitro* binding technique and the *in vivo* interaction(s) between Sry and Sip has yet to be established experimentally. Further, it is still uncertain if 1) the polymorphisms within the Q-rich domain alone, 2) polymorphisms and truncation of the Q-rich domain, or 3) a combination of these changes and additional genetic factors/modifiers are needed to manifest the incompatibility between the *domesticus* Sry and the B6 Sips. The eventual cloning and characterization of the *Sip* genes may provide clues to the validity of our hypothesis.

Eicher and her associates had introduced a B6 (129) type *Sry* transgene into the B6.Y^{Pos} mice and corrected the sex reversal

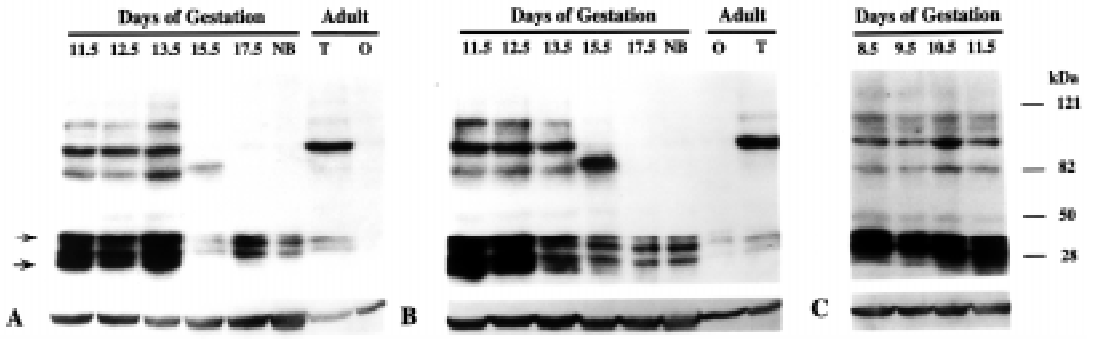

Fig. 10. Differential expression of Sips during embryo development. *Protein extracts from male* **(A)** *and female* **(B)** *embryos, excluding the gonads, at embryonic day 11.5, 12.5, 13.5, 15.5, 17.5, and newborns (NB) and adult testis (T) and ovary (O) controls were analyzed by farwestern blotting using B6Sry-Q probe. The Sips were detected most abundantly in embryos of both sexes at E11.5-E13.5 day. At E15.5 day, the 94 kDa Sip-1 was replaced by a new 85 kDa band which also disappeared at later stages of embryo development. The Sry-Q and Sip-2 and -3 interactions were more intense than that for Sip-1 in the fetal tissues (e.g., lanes 1-3 in A and B) while the reverse was true for adult testis (A, lane 7 and B, lane 8). Arrows indicate additional Sip bands detected by the B6Sry-Q probe in fetal tissue samples. Fetal tissues from embryos of mixed sexes at embryonic day 8.5, 9.5, 10.5 and 11.5 were further analyzed* **(C)**, *demonstrating the expression of Sips at these early stages of embryogenesis. Control western blots of the same (B and C) or identical (A) filters using anti-tubulin antibody are shown in the corresponding lower panels.*

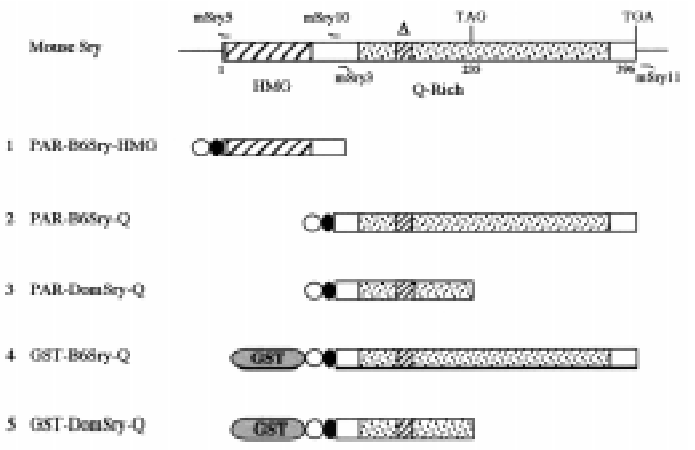

Fig. 11. Schematic representation of the fusion proteins of the mouse Sry. *The mouse Sry, top row, is an intronless gene and consists of an amino terminal domain containing a DNA-binding HMG box and a carboxyl Q-rich domain (Q-Rich). D identifies the polymorphic polyglutamine stretch that correlates to the B6.Y^{Dom} sex reversal (Coward et al., 1994). The amino acids are numbered according to the B6/129 Sry. The stop codon in the Sry of domesticus strains is denoted by TAG at codon 235. The stop codon for B6/129 Sry is denoted by TGA at codon 396. The positions of the PCR primers, mSry5 and mSry3 for the HMG box and mSry10 and mSry11 for the Q-rich domain, are marked. The fusion protein constructs (1) PAR-B6Sry-HMG, (2) PAR-B6Sry-Q and (3) PAR-DomSry-Q are expression cassettes for respective Sry fusion proteins in the pARΔR1 vector (Blanar and Rutter, 1992). The FLAG peptide and HMK signal are represented by open and filled circles respectively. The constructs (4) GST-B6Sry-Q and (5) GST-DomSry-Q are GST fusion proteins in pGEX2T and are derived from PAR-B6Sry-Q and PAR-DomSry-Q respectively.*

phenotype of these animals (Eicher *et al.*, 1995). This observation confirmed the notion that the differences between the B6 and *domesticus Sry* genes are responsible for the sex reversal in these animals. Sequence analysis of *Sry* genes from B6 and *domesticus* (Tirano, AKR and FVB) strains did not reveal any significant differences at their promoter (~5 kb) region (Carlisle *et al.*, 1996). Transgenic studies demonstrate that the *domesticus* (Tirano) *Sry* promoter is capable of directing the expression of a green fluorescent protein reporter gene in an identical pattern as that of the endogenous *Sry* gene in B6 mice (Xian *et al.*, submitted). On the other hand, the CAG repeats are highly polymorphic. Their encoded Q-rich domains correspond to the most significant differences between the B6 and *domesticus Sry* genes. We detected two slightly different binding patterns of Sips in adult testes from various mouse strains using the B6 and *domesticus* Sry-Q probes. The difference lies mainly in the sensitivity of each probe in detecting the 3 Sips. These observations correlate well with the fact that the B6.Y$^{Pos/Dom}$ animals are not completely sex-reversed. Even in the most affected cases, these animals develop either ovotestes or a combination of ovary and testis, indicating that testis determination and differentiation are still partially occurring during gonadogenesis. In fact, we did detect Sips in the sex-reversed gonads of adult B6.Y$^{Pos/Dom}$ animals. If the *poschiavinus* Sry Q-rich domain indeed interacts variably and inadequately with the B6 Sips, it would have explained the different forms of sex reversal in these animals.

The physiological significance of Sry-Sip interactions in the somatic cells of the adult testis and brain has yet to be elucidated. The detection of both normal and variant forms of Sips in ovotestes and XY ovaries of B6.Y^{Pos} animals suggests that these sex-reversed tissues still maintain certain degrees of testis-like expression profile. If Sry is required for spermatogenesis in adult, the Sip expression in somatic cells may signify their potential involvement in the same process.

Despite reports of low levels of Sry expression in two-cell and blastocyst stages of the mouse embryo (Zwingman *et al.*, 1993; Cao *et al.*, 1995), the high levels of Sips expression in non-gonadal tissues are not paralleled by elevated *Sry* expression (Hacker *et al.*, 1995; Jeske *et al.*, 1995). Further, the transient disappearance of a 94-kDa Sip-1 and appearance of an 85-kDa band in the fetal tissues at E15.5 day (Fig. 10A,B) indicate a differential regulation of the Sip expression during embryonic development. This shift of apparent molecular weight of Sip-1 is not observed in the fetal gonads. These findings suggest that the Sips, similar to other sex-determining genes such as *Wt-1* (Kreidberg *et al.*, 1993), *Sox9* (Kent *et al.*, 1996; Ng *et al.*, 1997) and *Sf-1* (Luo *et al.*, 1994), may be independently important for different aspects of embryonic development; apart from their role as co-factors in sex determination. Results from the present studies have provided a means to identify the *Sip* gene(s) and to characterize their diverse biological function(s).

Materials and Methods

Animals and tissues

Adult mice, C57BL/6J, 129-J, AKR/J, PL/J, FVB/NJ, SJL/J, *M. domesticus* (Tirano), and *M. poschiavinus* (Zalende) were obtained from The Jackson Laboratory (Bar Harbor, Maine). Timed pregnant Swiss Webster mice and adult Lewis rats were purchased from the Simonsen Laboratory (Gilroy, CA). Human testis samples were obtained from the Co-operative Human Tissue Network, Southwestern Division, Birmingham, AL. The chimpanzee testis was obtained from the Yerkes Regional Primates Center, Atlanta, GA. The wood lemming testis was obtained from a laboratory colony of Professor Karl Fredga, Uppsala University, Sweden. Tissue samples from dog, rabbit and guinea pig were supplied by Pel-Freeze Biologicals (Rogers, Arkansas).

Construction of fusion protein expression cassettes

PCR amplification was used to generate DNA fragments harboring coding sequences for the HMG box and Q-rich domain of the mouse Sry. The primers mSry5 5'-GGCCGAATTCATGGAGGGCCATGTCAAGCG-3' and mSry3 5'-GGCCGAATTCTCAGTACAGGTGTGCAGCTCTAC-3' were used to amplify the HMG box domain from the plasmid p422 that contains the *Sry* gene from strain 129 (Gubbay *et al.*, 1992). To generate a fragment containing the Q-domains of either the B6 or Dom (Tirano) *Sry* gene, primers mSry10 5'-GGCCGAATTCATCACATACAGGCAAGACTGGAG-3' and mSry11 5'-GGCCGAATTCAAGGGGGAGTGTTGGCATAGGTA-3' were used to amplify the corresponding coding regions from plasmids, p422 and pDomSry (Coward *et al.*, 1994), with the following conditions: 1x94°C 3 min, 30 cycles of 94°C 30 sec, 60°C 30 sec, 72°C 1 min, then 1x72°C 10 min. The PCR products were subcloned in-frame into the EocR1 site of pARΔR1 plasmid (Blanar and Rutter, 1992). The three expression cassettes are:

1) pAR-B6Sry-HMG (pPC2) consists of residues, 1-124, and the HMG box domain of B6 Sry.

2) pAR-B6Sry-Q (pPC34) consists of residues, 111-395, and the Q-rich domain of B6 Sry.

3) pAR-DomSry-Q (pPC35) consists of residues, 111-230, and the Q-rich domain of Dom (Tirano) Sry (Fig. 11).

Two additional expression cassettes containing the Q-rich domains of the B6 and Dom (Tirano) Sry were constructed with the pGEX2T plasmid

(Pharmacia, Uppsala, Sweden) by standard procedures. The additional fusion cassettes are:
4) GST-B6Sry-Q (pPC38) consists of residue #111-395 of B6 Sry, preceded by the GST and HMK signal peptide.
5) GST-DomSry-Q (pJZ1) consists of residue #111-230 of Dom (Tirano) Sry, preceded by GST and HMK signal peptide. All fusion proteins contain the heart muscle kinase (HMK) signal for *in vitro* labeling with HMK and [^{32}P]-γATP.

Production and labeling of recombinant fusion proteins

Fusion proteins were synthesized in bacterial host, BL21(DE3)LysS as described previously (Kaelin *et al.*, 1992; Ausubel *et al.*, 1995). The GST fusion proteins which offer a more convenient purification procedure were used in all experiments. They were labeled with [^{32}P]-γATP using HMK, extracted by glutathione Sepharose 4B binding and eluted by washing with 0.2 ml of 20 mM reduced glutathione (Kaelin *et al.*, 1992). The quality of the labeled proteins was assessed by SDS-PAGE and X-ray autoradiography.

Crude protein extract preparation

Tissues from adult mice and embryos from timed pregnant mice were dissected in phosphate buffered saline (PBS), quick-frozen on dry ice and stored at -80°C. For embryos at late stages, the testes and ovaries were dissected and pooled. Gonads at E11.5-13.5 day were dissected and stored separately for each embryo. Their sex was determined by PCR analysis of DNA from each embryo using the primers SryF3 (5'-AGAGACAAGTTTTGGGACTTGGTGAC-3') and SryB4 (5'-TAGCAA-GGGGGAGTGTTGGCATAG-3') from the *Sry* gene. Tissues were homogenized in cold PBS using a Polytron (Brinkman), and sucrose was then adjusted to 10%. After a low speed spin (1000xg), the supernatants were mixed with equal volume of 2X gel loading buffer (100 mM Tris-HCl, pH 6.8, 200 mM DTT, 4% SDS, 20% glycerol). Protein concentration of the prepared extracts was determined using a BCA protein assay kit (Pierce, Rockford, IL).

Preparation of crude nuclei and cytoplasm from cultured cells

(Ausubel *et al.*, 1995). Approximately 15 million cells were washed with cold PBS, resuspended in 0.8 ml of Buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 m PMSF) and homogenized in a Dounce homogenizer. The homogenization of the cells were monitored under a microscope to insure the nuclei were intact. The nuclei were collected by spinning at 10,000 rpm for 10 min at 4°C in an Eppendorf microcentrifuge. The supernatant was used as crude cytoplasmic extract. The nuclei were washed 3X with Buffer A, lysed by addition of loading buffer and processed for SDS-PAGE analysis.

Farwestern blotting

(Blanar and Rutter, 1992; Kaelin *et al.*, 1992). About 100 μg of protein extract per sample was size-fractionated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked sequentially for 60 min each time with 5% and 1% solution of dry milk in HEPES buffer (25 mM HEPES-KOH, pH 7.7, 25 mM NaCl, 50 mM $MgCl_2$). Hybridization was performed overnight at 4°C in Hyb75 (25 mM HEPES-KOH, pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% NP-40) with ^{32}P-labeled GST-fusion proteins at >200,000 cpm/ml. The filters were washed 3 times for 10 min each with 100-300 ml of Hyb75 buffer. All procedures were performed at 4°C with constant gentle shaking. For competition experiments, the respective recombinant proteins in bacterial extracts were estimated by SDS-PAGE and Comassie blue staining and protein standards. Bacterial lysates containing 10, 50 and 250 folds excess of the competitor proteins were incubated in the hybridization step in the farwestern blotting procedure. Specific binding was detected by X-ray autoradiography at -80°C. The apparent molecular weights of the bands were determined by comparing to prestained protein standards (Bio-Rad Laboratories) in the same gel.

Western blotting

To demonstrate even loading of samples, some farwestern blot filters were soaked in methanol and re-hydrated in water and PBS and processed for western blotting using a standard procedure. A monoclonal antibody against a tyrosinated tubulin peptide (Kreis, 1987) was used at a 1:1000 dilution (Sigma BioSciences, Clone TUB-1A2). The specific antibody binding was detected by a horse radish peroxidase-conjugated secondary antibody and enzymatically visualized using 4-chloro-1-naphthol as substrate. In all blots, a specific band of 55 kDa for the α-tubulin was consistently observed.

In situ protein blotting

In situ transfer of proteins from frozen sections of the mouse testis to PVDF membrane was performed as described by Okabe *et al.* (1993) with minor modifications. Briefly, mouse testes were dissected from adult animals, snap-frozen in liquid nitrogen and sectioned with a cryostat. Ten to 20 μm sections were picked up with a dry PVDF membrane and pre-wetted with methanol. The proteins from the sections were transferred to the PVDF membrane by placing the PVDF membrane on top of a small layer of 3 MM paper saturated with a transfer buffer (40 mM Tris- 20 mM HCl, 10% methanol) in a moist chamber for 10 min. Residual tissues were removed by gentle wiping with a Kimwipe under the blocking buffer. ^{35}S-labeled Sry protein probes were synthesized by *in vitro* transcription and translation using a TNT kit (Promega Inc.). The entire ORF or sequence coding for the Q-rich domain of the B6 Sr*y* were individually subcloned in pET15b plasmid (Novogen Inc.), linearized at the 3' end and subjected to *in vitro* transcription using T7 RNA polymerase. The resulting RNAs were then translated *in vitro* with rabbit reticulocyte lysate in the presence of 20 μCi of ^{35}S-methionine. The labeled proteins were then purified by Bio-spin 6 chromatography column (Bio-Rad Inc.) and used in farwestern blotting of the mouse testis section imprints on PVDF membrane. Specific Sry-Sip interactions were detected by X-ray autoradiography using β Max Hyperfilm (Amersham Inc.). After autoradiography, the tissue imprints were processed for immunostaining using either a monoclonal antibody against the tyrosinated a tubulin or a polyclonal antibody against the lactate dehydrogenase-X which stain specifically for the somatic and germ cells of the testis respectively. Both the Sry-Sip interaction and immunostaining were analyzed with a Zeiss Axiophot photomicroscope.

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