Identification of an interaction between SOX9 and HSP70

Owen J. Marshall, Vincent R. Harley*

Prince Henry's Institute of Medical Research, Monash Medical Centre, P.O. Box 5152, Melbourne, Vic. 3168, Australia

Received 12 February 2001; revised 26 March 2001; accepted 28 March 2001

First published online 20 April 2001

Edited by Gianni Cesareni

Abstract The campomelic dysplasia/autosomal sex reversal protein SOX9 is an important developmental transcription factor, required for correct bone and testis formation. Through in vitro and in vivo studies we have identified the heat shock protein HSP70 as an interacting partner for SOX9 in chondrocyte and testicular cell lines. HSP70 forms a ternary complex with DNA-bound SOX9. The interaction between HSP70 and SOX9 is ATP-independent and involves a highly conserved region of SOX9 hitherto of unknown function and the C-terminal region of HSP70. Our results implicate HSP70–SOX9 interactions in the assembly of multi-protein complexes during SOX9-mediated transcription.

Key words: Campomelic dysplasia; SOX9; HSP70; Protein–protein interaction

1. Introduction

SOX proteins are related to the mammalian testis-determining factor, SRY, via their HMG domain which binds and bends DNA [1,2]. The 65 kDa SOX protein, SOX9, binds a 10 base DNA consensus sequence and also possesses two transactivation domains at the C-terminal region of the protein, the PQS and PQA domains (for a review of SOX9, see [3,4]). In humans, haploinsufficiency of SOX9 causes campomelic dysplasia (CD), characterised by extreme skeletal and cartilage malformation, and male to female sex reversal in approximately 75% of XY individuals [5,6]. CD individuals also have defects in the brain, heart, lung and kidneys – suggesting the critical importance of SOX9 for correct development. Over the past 4 years, in vivo and in vitro studies have demonstrated that SOX9 regulates a number of genes in both chondrogenesis and testis formation. A firm link has been established between SOX9 and two important genes involved in chondrogenesis, namely type II collagen, *Col2a1* [7,8], and type XI collagen, *Col11a2* [9]. SOX9 appears to act together with two other proteins, L-SOX5 and SOX6, binding cooperatively to an enhancer region that is present in both genes [10]. Curiously, although SOX9 is the only member of the three SOX genes that possesses a potent transactivation domain, its presence on the enhancer element is necessary but not sufficient for gene transcription to occur [10]. Currently the mechanism by which the three proteins activate transcription is unknown, although the activation of the enhancer by SOX9 is phosphorylation state-dependent [11]. Recently another gene involved in chondrogenesis, *Aggrecan*, has also been implicated in regulation by SOX9 [12]. In the Sertoli cells of the mammalian testis, SOX9 activates the anti-Müllerian hormone (also known as Müllerian inhibiting substance) gene, *AMH*. There exist conserved binding sites for SOX9, SF-1 (or steroidogenic factor 1) and GATA-4 in the minimal *AMH* promoter [13,14], and protein–protein interactions between SOX9 and SF-1 (13), SF-1 and the Wilms' tumour protein, WT1 [15], and SF-1 and GATA-4 [16] all contribute to the activation of the *AMH* gene. However, knock-in mutations of the SOX9 and SF-1 binding sites of the *AMH* promoter in mice have shown that only by ablating the SOX9 binding site is it possible to completely eliminate *AMH* activation [17].

The ubiquitous HSP70 chaperones represent a family of heat shock proteins of approximately 70 kDa in molecular weight. The HSP70 family are involved in the ATP-dependent refolding of denatured proteins following heat stress (reviewed in [18]). However, HSP70 is also implicated in a number of completely unrelated cellular processes. These include uncoating clathrin pits on the cell membrane [19], the activation of the steroid hormone complexes (reviewed in [20]), and a number of roles in DNA replication and gene transcription. It is now thought that HSP70 is involved in transcriptional activation by the glucocorticoid receptor (GR) since the chaperone is tightly bound to the GR:glucocorticoid response element complex [21]; and levels of nuclear HSP70 regulate the DNA binding activity of the AP-1 transcription factor complex [22]. Interestingly, an association between the bacterial homologue of HSP70, DnaK, and viral DNA replication has long been established [23], where DnaK appears to function by aiding the correct association of proteins within a multi-protein complex. This has also been observed with the human papillomavirus in eukaryotes [24] suggesting that a role of HSP70 in multi-protein complex formation has been conserved. Most significantly for this study, though, is the observation of a strong interaction between WT1 and HSP70 that has been shown by some elegant in vivo studies to be vital for the correct control of cell cycle by WT1 [25] although the exact mechanism remains unknown.

Considering that WT1 is also involved in *AMH* activation during vertebrate sex determination, we were interested to determine whether WT1 and/or its closely interacting factor HSP70 could interact with SOX9. Here we present evidence

*Corresponding author. Fax: (61)-3-9594 6125.
E-mail: vincent.harley@med.monash.edu.au

PII: S0014-5793(01)02407-3

that HSP70 also interacts with SOX9 in an ATP-independent fashion, and that this interaction may have implications on SOX9-mediated transactivation, not only in the field of sex determination, but in all transcriptional processes controlled by SOX9.

2. Materials and methods

2.1. Production of wild-type full-length SOX9 and deletion mutants

HA-tagged full-length SOX9 and deletion mutants SOX9(ΔPQA) and SOX9(1–235) were produced as described previously [26]. Deletion mutant SOX9(1–400) was produced through a *Hin*dIII/*Pvu*II digest of the vector pBS-HA-SOX9 [26] ligated into a *Hin*dIII/*Eco*RV digest of pcDNA3 (Invitrogen). Proteins were produced in vitro using a TNT kit (Promega). A small proportion of each sample was translated with the incorporation of [^{35}S]methionine to verify that proteins of the correct molecular mass had been translated. SOX9 HMG box was expressed recombinantly in *Escherichia coli* and purified as described previously [27].

2.2. Electromobility shift assay (EMSA)

The sequence of the upper strand used for the oligonucleotide probes (SOX9CON) was GGGTTAAC<u>AG</u>**AACAAT**<u>GG</u>AATCT-GGT-AGA. The sequence of the SOX binding site is shown in bold type; flanking residues giving additionally specific SOX9 binding are underlined [27]. Probes were labelled as described previously [27]. EMSA was carried out as described in [27] with the following modifications: in vitro translated proteins and HSP70 were incubated at 4°C for 15 min in 12 μl total of binding buffer, then for an additional 20 min at 4°C following the addition of ^{33}P-labelled DNA (~0.4 nM). Antibodies raised against HSP70 were pre-incubated with the HSP70 protein at room temperature for 15 min prior to addition to the reaction mixture. Protein–DNA complexes were resolved on 6% non-denaturing polyacrylamide gels run at 350 V (~35 mA) for 1.5 h. Gels were visualised and complexes were quantitated by PhosphorImager analysis (Fuji). When ATP or ADP was added to the reaction mixture, 10 mM $MgCl_2$ and 1 mM dithiothreitol were also present in the binding buffer.

2.3. Western blot analysis

Protein samples were blotted on Hi-Bond nitrocellulose membrane (Amersham) and detected as described previously [8], using 1:1000 or 1:2000 dilutions of primary antibodies and 1:1000 or 1:2000 dilutions of horseradish peroxidase-conjugated secondary antibodies. Blots were visualised by the ECL chemiluminescence detection kit (Amersham).

2.4. Immunoprecipitation

Whole cell extracts made from RCS cells (2.5 mg) were incubated for 2 h at 4°C with 1 μg of an α-SOX9 primary antibody or an irrelevant antibody control, prior to the addition of protein A agarose (Amersham) and subsequent rotation at 4°C overnight. Protein A immunoprecipitates were centrifuged and washed three times in TBST buffer, before being boiled in 2×Laemmli buffer.

2.5. Cell types and culture

The testicular teratocarcinoma cell line, NTera2/D1, and the rat chondrosarcoma cell line, RCS, cells were cultured as a monolayer in Dulbecco's modified Eagle's medium, supplemented with 1% (v/v) penicillin/streptomycin, 1% (v/v) L-glutamine and 10% (v/v) foetal calf serum at 37°C under 5% CO_2.

2.6. Immunocytochemistry

Cells were grown in chamber slides for 24–48 h, washed three times in phosphate-buffered saline (PBS) and fixed in 2% paraformaldehyde in PBS for 30 min. Following washing in PBS, cells were permeabilised in 0.5% Nonidet P40 in PBS for 5 min with agitation, washed, and incubated in blocking buffer (5% goat and 5% horse serum diluted in incubation buffer (1 mM $CaCl_2$, 3% globulin-free bovine serum albumin, 0.5% Triton X-100 in PBS)) for 30 min at room temperature. Slides were washed and then incubated overnight in a 1:100 dilution of SAM α-SOX9 and N15 α-HSP70 antibodies in incubation buffer overnight at 4°C. Following washing, slides were incubated in 1:200 dilutions of FITC-conjugated anti-rabbit and Texas red-conjugated anti-mouse secondary antibodies (Vector) in incubation buffer at room temperature for 2 h. Slides were washed and visualised under a Leica fluorescent microscope or a Leica laser-scanning confocal microscope.

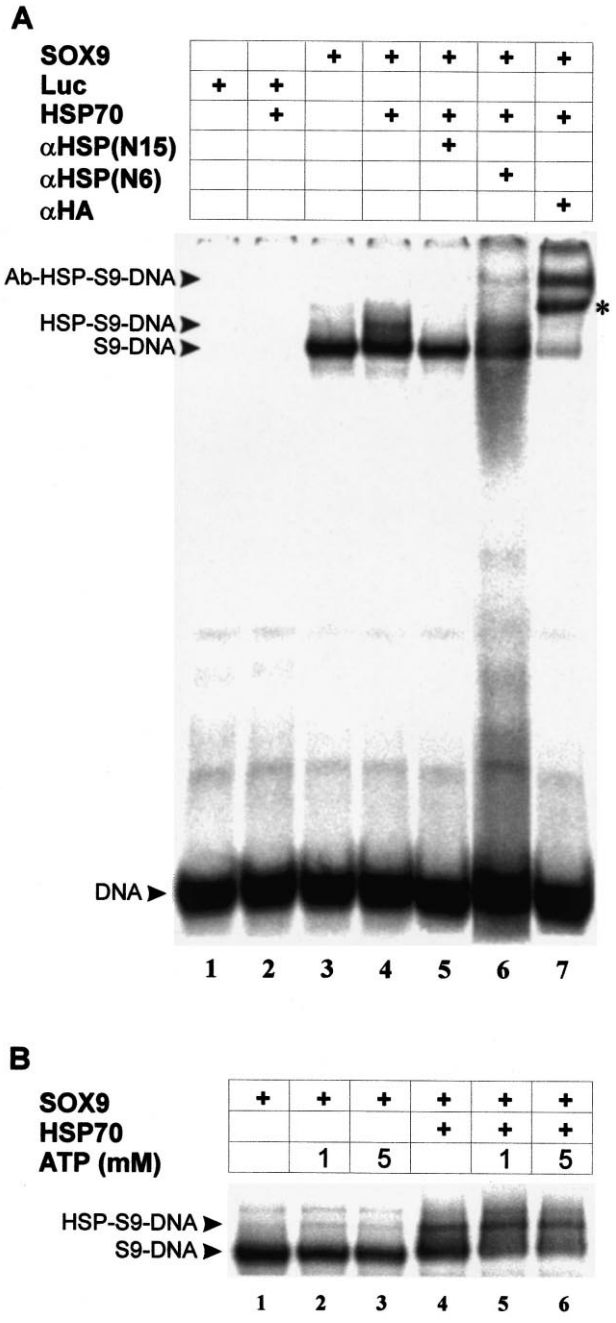

Fig. 1. HSP70 forms a ternary complex with SOX9 and DNA, independent of ATP concentration. A: In an EMSA reaction, in vitro translated human HA-tagged SOX9, and recombinant human HSP72 or partially purified mouse Hsp73 were incubated together prior to the addition of a ^{33}P-labelled DNA probe. The anti-HSP70 antibodies were pre-incubated with HSP72 or Hsp73 prior to being added to the reaction mixture. Lane 6 contains the addition of a complete protease inhibitor mix. In vitro translated luciferase was used as a control (lanes 1 and 2). The asterisk in lane 7 indicates the presence of an antibody–SOX9–DNA species. B: In an EMSA reaction, in vitro translated SOX9 was incubated with either 0, 1 or 5 mM ATP, with (lanes 4–6) and without (lanes 1–3) the presence of HSP70 in the reaction mix. The slowly migrating band above the HSP70–SOX9–DNA complex is possibly artefactual SOX9 dimer as observed previously [26].

2.7. Bioinformatic tools

Secondary structure for the SOX9 protein was predicted via the Predict Protein Server based on the PHD analysis algorithms [28]. Sequence alignments were performed using the ClustalW algorithm [29] and displayed using the ES-Pript server [30].

3. Results

To test a possible interaction between HSP70 and SOX9, HSP70 was incubated with the SOX9–DNA complex run on EMSA gels, using in vitro translated human SOX9 and recombinant human HSP70 (Fig. 1A). The addition of 1–2 μM of HSP70 caused the appearance of a band that migrated more slowly than the SOX9–DNA complex (lane 4), suggesting that a ternary complex was formed between HSP70, SOX9 and DNA. Both the inducible and cognate isoforms of HSP70 were able to form the ternary complex, suggesting that complex formation was not specific to a particular HSP70 isoform (data not shown). It should be noted that the concentration of HSP70 used in this system is comparable with the concentrations required to interact with the AP-1 transcription factor complex in vitro (0.7–1.4 μM) [22] and for maximal steroid hormone foldosome assembly with the glucocorticoid receptor in vitro (~3 μM) [31]. Unlike with AP-1, HSP70 appears to have no effect upon the DNA binding activity of SOX9. To determine whether the putative HSP70–SOX9–DNA complex did indeed contain HSP70 and SOX9, antibodies raised against both HSP70 and the HA tag of SOX9 were pre-incubated with the reaction mixture. Addition of an HSP70 antibody (clone N6) resulted in a supershift of the HSP70–SOX9–DNA complex (lane 6), while the addition of an antibody raised against the HA tag of the in vitro translated SOX9 caused a supershift of both the SOX9–DNA (asterisk) and HSP70–SOX9–DNA complexes (lane 7). Taken together, these results suggest that HSP70 can recognise DNA-bound SOX9. Anti-HSP70 antibodies have been raised which recognise different domains of HSP70. Clone N6 recognises an epitope between amino acids 373–430 of the HSP70 substrate binding domain (data not shown and R. Anderson, personal communication). The binding of N6 to HSP70 had no effect upon the HSP70–SOX9 interaction as revealed by the supershift (lane 6). In contrast, a second HSP70 antibody, clone N15, recognises an epitope between amino acids 540 and 650 of the HSP70 C-terminus (data not shown and R. Anderson, personal communication). Pre-incubation of HSP70 with N15 specifically blocked the interaction between HSP70 and SOX9 (lane 5), implying that the region of HSP70 recognised by N15 (amino acids 540–650) is also involved in SOX9 binding.

Because of the inherent ability of heat shock proteins to bind to any denatured or incorrectly folded substrate in an ATP-dependent manner, it was investigated whether the interaction observed between HSP70 and SOX9 arose merely through the binding of HSP70 to denatured or incorrectly folded SOX9 caused by the in vitro translation process. The ability of HSP70 to bind to denatured or incorrectly folded proteins is known to be dependent upon ATP/ADP concentrations – at high (>1 mM) concentrations of ATP, HSP70 fails to bind to a hydrophobic substrate, whereas an increase in ADP concentrations will cause a stronger binding of HSP70 to the substrate [32,33]. However, although very high concentrations of ATP were observed to cause a gradual reduction in SOX9–DNA binding, increasing ATP concentrations to 5 mM was not observed to cause a reduction in the formation of the HSP70–SOX9 multiprotein complex at 4°C (Fig. 1B) or at 37°C (data not shown). Furthermore, substituting 1 mM ADP into the reaction mixture was also observed to have no effect on complex formation (data not shown). We conclude that the interaction between HSP70 and SOX is not dependent on ATP or ADP concentrations, and thus not likely to be an artefact resulting from the binding of HSP70 to denatured protein.

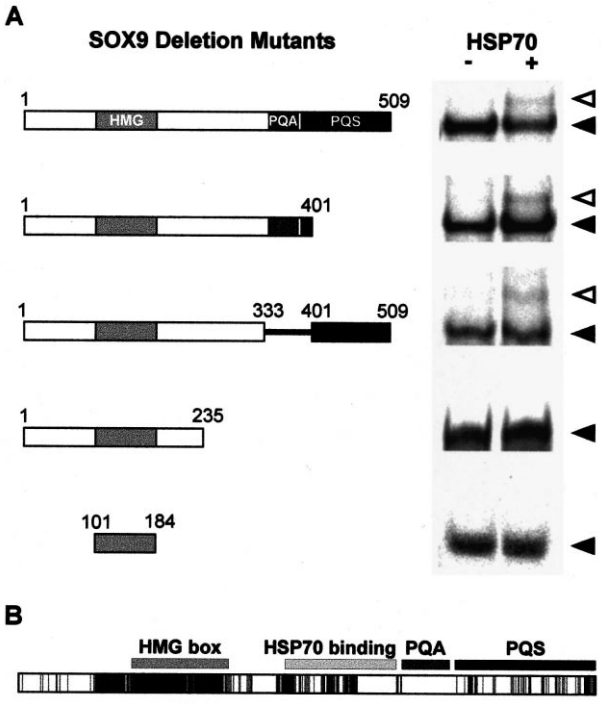

Fig. 2. Identification of the HSP70 interacting region of SOX9. A: Deletion analysis of the interaction between SOX9 and HSP70. In EMSA reactions, the following proteins were analysed for an interaction with HSP70: full-length SOX9; SOX9(1–401); SOX9(Δ333–401); SOX9(1–235); purified SOX9 HMG box (101–184). Proteins were incubated with the DNA alone (−) or following pre-incubation with HSP70 (+). Black arrowheads show the position of the SOX9–DNA band; white arrowheads mark the presence of the HSP70–SOX9–DNA complex if present. B: Conservation of the HSP70 interacting region of SOX9. SOX9 from nine vertebrate species (human – accession number P48436; pig – O18896; mouse – S52469; chicken – P48434; alligator – Q9YGP7; frog – BAA95427; trout – O57395; zebrafish – AAG 09814, turtle – [41]) SOX8 from three species (human – AF228664; mouse – AW244736; chicken – AF228664) and SOX10 from four species (human – XM009977; rat – NM019193; chicken – AF152356; mouse – AF017182) were aligned using ClustalW; residues completely conserved across the three proteins are represented by a black line; homologous substitutions are represented by a stippled line. The HMG box, PQS and PQA domains and HSP70 interacting region are boxed.

To identify the region of SOX9 that interacts with HSP70, the HSP70 binding ability of a number of deletion mutants of SOX9 was tested by EMSA (Fig. 2A). Deletions spanning most of the PQS domain (Δ401–509) or the PQA domain (Δ333–402) both retained the ability to bind HSP70. In contrast, neither purified SOX9 HMG box (100–180) nor the N-terminal half of the protein (1–235) exhibited any HSP70 binding activity. The region of SOX9 that interacts with HSP70 thus appears to lie within a stretch of 96 amino acids between residues 236 and 332 – a region of the protein that

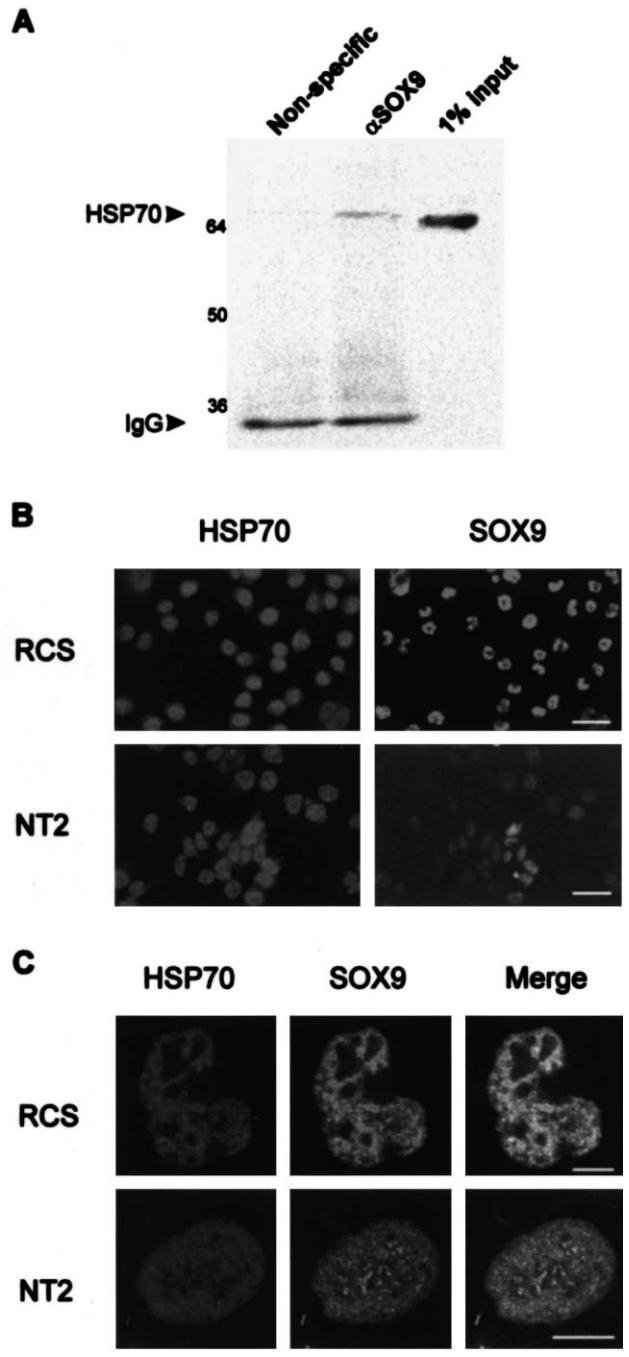

Fig. 3. Characterisation of the interaction between SOX9 and HSP70 in vivo. A: Co-immunoprecipitation of HSP70 with SOX9. Whole cell extracts from RCS cells (2.5 mg) were immunoprecipitated with either anti-SOX9 or irrelevant antibodies. Samples were washed and analysed via SDS–PAGE and immunoblotting with an anti-HSP70 antibody. A proportion of the whole cell lysate was included as a positive control. Molecular mass markers (in kDa) are indicated to the left of the figure. B: Localisation of SOX9 and HSP70 to the nucleus of RCS and NT2/D1 cell lines. Immunocytochemical staining of HSP70 in red and SOX9 in green as visualised under a fluorescent microscope. Scale bar: 50 μm. C: Localisation of SOX9 and HSP70 within the nucleus of RCS and NT2/D1 cell lines. Immunocytochemical staining of SOX9 in green, HSP70 in red or both as visualised under a confocal laser scanning microscope. Scale bar: 5 μm.

has no previously known function and which is highly conserved in both SOX9 and the two related SOX proteins, SOX8 and SOX10 (Fig. 2B).

Since an interaction between HSP70 and SOX9 was demonstrated in vitro by EMSA, it was investigated whether such an interaction could be supported by co-immunoprecipitation of SOX9 from cultured cells. Immunoprecipitates of SOX9 within whole cell extracts from a rat chondrosarcoma line, RCS, were observed to contain a 70 kDa band, detectable with an α-HSP70 antibody (N6) that co-migrated with HSP70 found in the extract (Fig. 3A). In contrast, HSP70 was not found to co-precipitate with a non-specific rabbit polyclonal antibody (raised against FD-bacteriophage). Attempts to perform the reverse experiment were unsuccessful owing to a failure to immunoprecipitate HSP70. This evidence thus supports the above in vitro studies, and suggests that SOX9 and HSP70 interact under physiologically relevant conditions found in mammalian cells in vivo.

In order to determine whether the two proteins co-localised in vivo, we examined the distribution of HSP70 and SOX9 in mammalian cell lines. Both proteins were found to exhibit strong nuclear staining in two cell lines known to express SOX9, the chondrocyte cell line RCS and a human Sertoli-like teratocarcinoma cell line, NTera2/D1 (NT2), using immunocytochemistry (Fig. 3B). In the RCS cell line, both HSP70 and SOX9 exhibited punctate co-localisation within the nucleus, but not the nucleoli, of RCS cells (Fig. 3C). Curiously, in the NT2 cell line SOX9 exhibited defined punctate staining within the cell nucleoli as well, an observation which has not been described previously and which was not seen with HSP70 (Fig. 3C). While both proteins showed some co-localisation outside the nucleoli, this was not to the same degree as that observed with the RCS cell line and raises the possibility that other interacting factors may mediate the interaction in this cell line. No background immunofluorescence was detectable in cells incubated without primary antibodies (data not shown). This result thus supports the earlier in vitro and co-immunoprecipitation studies, and suggests that SOX9 and HSP70 may be present within the nuclei of SOX9 expressing cells as part of a multi-protein complex.

4. Discussion

The results described above present strong evidence for an interaction between SOX9 and HSP70. The two proteins were found to interact as part of a multi-protein complex on DNA, through a specific interaction that did not appear to be through the process of HSP70 binding to denatured or incorrectly folded SOX9 protein. That the in vitro association between SOX9 and HSP70 may have a physiological basis can be seen through two results. Namely, that HSP70 was found to co-immunoprecipitate with SOX9 protein, and that the two proteins exhibited strong co-localisation within the nuclei of a SOX9-expressing cell line. While SOX9 is well established as an important developmental protein, HSP70 also exhibits tissue-specific expression during development. The heat shock protein co-localises to urogenital tissue that also expresses WT1 [25], suggesting that HSP70 should be expressed in the developing Sertoli cells that express both WT1 and SOX9. Furthermore, during endochondral bone formation HSP70 mRNA is developmentally expressed weakly in the resting and strongly in the proliferative phases of chondrogenesis

[34] – the two stages of chondrogenesis in which type II collagen and SOX9 mRNA are most strongly expressed [10,35]. In contrast, HSP70 mRNA is absent in hypertrophic chondrocytes [34] which also have little or no SOX9 expression [35]. The reason for the presence of HSP70 during this early stage of chondrogenesis is unknown, and we suggest that one reason may be a specific role in SOX9-mediated gene activation. Such evidence implies a strong link between SOX9 and HSP70 expression during development.

Through the use of deletion mutants of SOX9, it has been determined that HSP70 interacts with a 96 amino acid stretch of the SOX9 protein – a region which lies outside both the DNA binding and transactivation domains of SOX9. The region that interacts with HSP70 is highly conserved, both in SOX9 and in the two related SOX genes, SOX8 and SOX10. However, this region exhibits little homology to any other protein outside the SOX transcription factors. We suggest that an interaction with HSP70 has been one reason behind such a high degree of conservation in this region, and that it is highly probable that both SOX8 and SOX10 will also interact with HSP70. The ability of a specific anti-HSP70 antibody to block the interaction between HSP70 and SOX9 suggested that SOX9 binds to the carboxy-terminus of the heat shock protein, between amino acids 540 and 650. This α-helical domain of HSP70 is a region that appears to be involved in protein–protein interactions, as it is already known to interact with the proteins Hip [36], Hop (or p60) [37] and Chip [38]. These interact with HSP70 via a tetratricopeptide repeat (or TPR) domain – a 34 amino acid domain that consists of a helix-turn-helix motif [39]. However, analysis of the secondary structure of the HSP70 binding region of SOX9 using the PredictProtein server [28] showed the chance of any helical structure being present within this region is extremely low. We therefore suggest that SOX9 interacts with the C-terminus of HSP70 via a novel motif.

Although some sequence specificity within SOX proteins may exist [27], protein–protein interactions are likely to be vital for the specific regulation of different genes in different tissues by the SOX transcription family [40]. SOX9, in particular, is known to form part of a greater multi-protein complex for the regulation of both the collagen genes and *AMH*. It is possible that an interaction with HSP70 in this context may aid the correct association of SOX9 with other proteins upon these regulatory elements. With respect to viral DNA replication, HSP70 appears to play a vital role in governing the correct formation of multi-protein complexes [23,24], and we suggest that such a mechanism may also be employed in SOX9-mediated gene regulation. Another, more specific way in which an interaction between HSP70 and SOX9 may be significant can be seen in the process of mammalian sex determination. The regulation of the crucial *AMH* gene is controlled not only by SOX9 and SF1 [13], but also by WT1 [15]. However, while binding sites to both SOX9 and SF1 are conserved within the *AMH* promoter, WT1 has no conserved binding site and it interacts with SF1 only very weakly. Considering that WT1 strongly interacts with HSP70 [25] it is possible to speculate that WT1 binding at the *AMH* promoter is stabilised by the formation of a SOX9–HSP70–WT1 protein complex. The fact that SOX9 and SF1 also interact raises the possibility that the four proteins may form a tightly associated complex at this promoter. In conclusion, we describe here an interaction between SOX9 and HSP70 that occurs while SOX9 is bound to DNA and may have implications for the control of gene regulation by SOX9 during development.

Acknowledgements: We thank William Welch and Robin Anderson for the kind gift of the anti-HSP70 antibodies N6 and N15. We also thank Dean Naylor for the gift of mouse Hsc73, and Michael Clarkson, Peter Fuller and Wah-Chin Boon for helpful comments on the manuscript and Ms Sue Panckridge for artwork. This work was supported by Australian National Health and Medical Research Council Block Grant 983001.

References

[1] Harley, V.R., Jackson, D.I., Hextall, P.J., Hawkins, J.R., Berkovitz, G.D., Sockanathan, S., Lovell-Badge, R. and Goodfellow, P.N. (1992) Science 255, 453–456.
[2] Pontiggia, A., Rimini, R., Harley, V.R., Goodfellow, P.N., Lovell-Badge, R. and Bianchi, M.E. (1994) EMBO J. 13, 6115–6124.
[3] Koopman, P. (1999) Cell. Mol. Life Sci. 55, 839–856.
[4] Marshall, O.J. and Harley, V.R. (2000) Mol. Genet. Metab. 71, 455–462.
[5] Foster, J.W., Dominguez-Steglich, M.A., Guioli, S., Kowk, G., Weller, P.A., Stevanovic, M., Weissenbach, J., Mansour, S., Young, I.D., Goodfellow, P.N. and Schafer, A.J. (1994) Nature 372, 525–530.
[6] Wagner, T., Wirth, J., Meyer, J., Zabel, B., Held, M., Zimmer, J., Pasantes, J., Bricarelli, F.D., Keutel, J., Hustert, E., Wolf, U., Tommerup, N., Schempp, W. and Scherer, G. (1994) Cell 79, 1111–1120.
[7] Bell, D.M., Leung, K.K., Wheatley, S.C., Ng, L.J., Zhou, S., Ling, K.W., Sham, M.H., Koopman, P., Tam, P.P. and Cheah, K.S. (1997) Nature Genet. 16, 174–178.
[8] Lefebvre, V., Huang, W., Harley, V.R., Goodfellow, P.N. and de Crombrugghe, B. (1997) Mol. Cell. Biol. 17, 2336–2346.
[9] Bridgewater, L.C., Lefebvre, V. and de Crombrugghe, B. (1998) J. Biol. Chem. 273, 14998–15006.
[10] Lefebvre, V., Li, P. and de Crombrugghe, B. (1998) EMBO J. 17, 5718–5733.
[11] Huang, W., Zhou, X., Lefebvre, V. and de Crombrugghe, B. (2000) Mol. Cell. Biol. 20, 4149–4158.
[12] Sekiya, I., Tsuji, K., Koopman, P., Watanabe, H., Yamada, Y., Shinomiya, K., Nifuji, A. and Noda, M. (2000) J. Biol. Chem. 275, 10738–10744.
[13] de Santa Barbara, P., Bonneaud, N., Boizet, B., Desclozeaux, M., Moniot, B., Sudbeck, P., Scherer, G., Poulat, F. and Berta, P. (1998) Mol. Cell. Biol. 18, 6653–6665.
[14] Watanabe, K., Clarke, T.R., Lane, A.H., Wang, X. and Donahoe, P.K. (2000) Proc. Natl. Acad. Sci. USA 97, 1624–1629.
[15] Nachtigal, M.W., Hirokawa, Y., Enyeart-Van Houten, D.L., Flanagan, J.N., Hammer, G.D. and Ingraham, H.A. (1998) Cell 93, 445–454.
[16] Tremblay, J.J. and Viger, R.S. (1999) Mol. Endocrinol. 13, 1388–1401.
[17] Arango, N.A., Lovell-Badge, R. and Behringer, R.R. (1999) Cell 99, 409–419.
[18] Mayer, M.P., Schroder, H., Rudiger, S., Paal, K., Laufen, T. and Bukau, B. (2000) Nature Struct. Biol. 7, 586–593.
[19] Jiang, R.F., Greener, T., Barouch, W., Greene, L. and Eisenberg, E. (1997) J. Biol. Chem. 272, 6141–6145.
[20] Pratt, W.B., Gehring, U. and Toft, D.O. (1996) Exs 77, 79–95.
[21] Srinivasan, G., Post, J.F. and Thompson, E.B. (1997) J. Steroid Biochem. Mol. Biol. 60, 1–9.
[22] Carter, D.A. (1997) FEBS Lett. 416, 81–85.
[23] Dodson, M., Echols, H., Wickner, S., Alfano, C., Mensa-Wilmot, K., Gomes, B., LeBowitz, J., Roberts, J.D. and McMacken, R. (1986) Proc. Natl. Acad. Sci. USA 83, 7638–7642.
[24] Liu, J.S., Kuo, S.R., Makhov, A.M., Cyr, D.M., Griffith, J.D., Broker, T.R. and Chow, L.T. (1998) J. Biol. Chem. 273, 30704–30712.
[25] Maheswaran, S., Englert, C., Zheng, G., Lee, S.B., Wong, J., Harkin, D.P., Bean, J., Ezzell, R., Garvin, A.J., McCluskey, R.T., DeCaprio, J.A. and Haber, D.A. (1998) Genes Dev. 12, 1108–1120.

[26] McDowall, S., Argentaro, A., Ranganathan, S., Weller, P., Mertin, S., Mansour, S., Tolmie, J. and Harley, V. (1999) J. Biol. Chem. 274, 24023–24030.
[27] Mertin, S., McDowall, S.G. and Harley, V.R. (1999) Nucleic Acids Res. 27, 1359–1364.
[28] Rost, B. (1996) Methods Enzymol. 266, 525–539.
[29] Thompson, J.D., Higgins, D.G. and Gibson, T.J. (1994) Nucleic Acids Res. 22, 4673–4680.
[30] Gouet, P., Courcelle, E., Stuart, D.I. and Metoz, F. (1999) Bioinformatics 15, 305–308.
[31] Hutchison, K.A., Dittmar, K.D., Czar, M.J. and Pratt, W.B. (1994) J. Biol. Chem. 269, 5043–5049.
[32] Palleros, D.R., Welch, W.J. and Fink, A.L. (1991) Proc. Natl. Acad. Sci. USA 88, 5719–5723.
[33] Palleros, D.R., Shi, L., Reid, K.L. and Fink, A.L. (1994) J. Biol. Chem. 269, 13107–13114.
[34] Otsuka, G., Kubo, T., Imanishi, J. and Hirasawa, Y. (1996) Nippon Geka Hokan 65, 39–48.
[35] Ng, L.J., Wheatley, S., Muscat, G.E., Conway-Campbell, J., Bowles, J., Wright, E., Bell, D.M., Tam, P.P., Cheah, K.S. and Koopman, P. (1997) Dev. Biol. 183, 108–121.
[36] Hohfeld, J., Minami, Y. and Hartl, F.U. (1995) Cell 83, 589–598.
[37] Smith, D.F., Sullivan, W.P., Marion, T.N., Zaitsu, K., Madden, B., McCormick, D.J. and Toft, D.O. (1993) Mol. Cell. Biol. 13, 869–876.
[38] Ballinger, C.A., Connell, P., Wu, Y., Hu, Z., Thompson, L.J., Yin, L.Y. and Patterson, C. (1999) Mol. Cell. Biol. 19, 4535–4545.
[39] Das, A.K., Cohen, P.W. and Barford, D. (1998) EMBO J. 17, 1192–1199.
[40] Kamachi, Y., Cheah, K.S. and Kondoh, H. (1999) Mol. Cell. Biol. 19, 107–120.
[41] Spotila, L., Spotila, J.R. and Hall, S.E. (1999) J. Exp. Zool. 281, 417–427.