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Heat Shock Protein 2 Promoter Drives Cre Expression in Spermatocytes of Transgenic Mice

Amy L. Inselman^{1,2,4}, Noriko Nakamura^{1,3,4}, Paula R. Brown¹, William D. Willis¹, Eugenia H. Goulding¹, and Edward M. Eddy¹

¹Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

Summary

We generated transgenic mouse line C57BL/6-*Tg(Hspa2-cre)1Eddy/J* (*Hspa2-cre*), which expresses cre-recombinase under the control of a 907-bp fragment of the heat shock protein 2 (*Hspa2*) gene promoter. Transgene expression was determined using *Gt(ROSA)26Sor^{tm1Sor}/J* (ROSA26) and *Tg(CAG-Bgeo/GFP)21Lbe/J* (Z/EG) reporter strains and RT-PCR and immunohistochemistry assays. *Hspa2-cre* expression mimicked the spermatogenic cell-specific expression of endogenous HSPA2 within the testis, being first observed in leptotene/zygotene spermatocytes. Expression of the transgene also was detected at restricted sites in the brain, as occurs for endogenous HSPA2. Although the results of mating the *Hspa2-cre* mice to mice with a floxed *Cdc2a* allele indicated that some expression of the transgene occurs during embryogenesis, the *Hspa2-cre* mice provide a valuable new tool for assessing the roles of genes during and after meiotic prophase in pachytene spermatocytes.

INTRODUCTION

Spermatogenesis is a complex, coordinated developmental process characterized by mitotic proliferation of spermatogonia, dramatic transformation in nuclear content and chromatin organization in meiotic spermatocytes, and differentiation of postmeiotic haploid spermatids into spermatozoa capable of fertilization. The use of targeted mutagenesis in embryonic stem cells to generate gene knockout mice has contributed significantly to understanding how these processes are regulated. However, this approach has been limited to genes that are not also essential during development or for maintenance of viability during fetal and adult life. Use of cre-*LoxP* recombinase technology has overcome this problem for many systems, but the lack of appropriate cre-expressing mice has limited studies of genes involved in regulating cell cycle processes or other essential cellular functions during meiosis in the male. Transgenic mice exist in which cre is driven by promoters from the synaptonemal complex protein 1 (*Sycp1*) (Vidal *et al*, 1998) or phosphoglycerate kinase 2 (*Pgk2*) (Ando *et al*, 2000; Bhullar *et al*, 2001) genes, but weak and/or aberrant excision (Rassoulzadegan *et al*, 2002; Rasoulpour and Boekelheide, 2006; Ando *et al*, 2000; Bhullar *et al*, 2001) considerably limits their usefulness for studying the roles of most genes during meiosis in males.

Correspondence and requests for materials should be addressed to: E. M. Eddy, NIEHS, NIH, Research Triangle Park, North Carolina 27709. eddy@niehs.nih.gov.

²Current address: Division of Personalized Nutrition and Medicine, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, Arkansas 72079

³Current address: Department of Pharmacology, Physiology and Toxicology, BBSC Marshall University, Huntington, WV 25755

⁴These authors contributed equally to this work.

The *Hspa2* gene encodes a member of the HSP70 family of heat-shock proteins that serve as molecular chaperones. Unlike most other members of the HSP70 family, *Hspa2* is developmentally regulated and expressed predominantly in spermatocytes and spermatids (Rosario *et al*, 1992; Dix *et al*, 1996a; Eddy, 1999). Translation occurs immediately after transcription in leptotene/zygotene spermatocytes and the HSPA2 protein is present at high levels in primary spermatocytes (O'Brien, 1987; Dix *et al*, 1997). In addition, previous studies had characterized the *Hspa2* gene promoter in transgenic mice using *LacZ* as the reporter (Dix *et al*, 1996b). This led us to generate an *Hspa2*-cre line that expresses cre in spermatocytes to overcome the limitations of the other transgenic lines.

RESULTS AND DISCUSSION

A 907-bp region of the mouse *Hspa2* gene proximal promoter was used for constructing the *Hspa2*-cre transgene (Fig. 1a). It contained a 640-bp fragment 5' to the *Hspa2* transcription start codon shown previously to contain the minimal promoter region sufficient to drive expression of a *lacZ* transgene in spermatocytes (Dix *et al*, 1996b). In an attempt to reduce positional effects on transgene expression, we added to each end of the transgene a 53-bp region of the SP-10 (acrosomal vesicle protein 1, *Acrv1*) spermatid-specific promoter that had been shown to function as an insulator in somatic cells (Reddi *et al*, 2003). Additional studies will be required to determine if the addition of insulators was beneficial.

Pronuclear microinjection resulted in the production of seven independent transgenic lines carrying the *Hspa2*-cre transgene. Founders were identified by PCR and Southern analysis of genomic DNA (Fig. 1b and data not shown). Four of the lines efficiently transmitted the transgene and line 54 was selected for further analysis based on the level and tissue specificity of transgene expression seen in preliminary studies. Transcriptional activity of the *Hspa2*-cre transgene was assessed by RT-PCR using cDNA from brain, heart, liver, kidney, spleen, testis, and ovary. Expression of the *Hspa2*-cre transgene was robust in testis and detectable in brain (Fig. 1c), consistent with previous RT-PCR results for *Hspa2* expression (Dix *et al*, 1996b).

The ROSA26 reporter strain was used to verify that *Hspa2*-cre activity was targeted to spermatogenic cells in the testis. Tissues were collected from postnatal day 20 *Hspa2*-cre;ROSA26 compound transgenic males and wild type littermates (Fig. 2). In the presence of cre activity, the *loxP*-flanked DNA STOP sequence located within the *lacZ* gene of ROSA26 reporter mice is excised and *lacZ* expression is detected by X-gal staining (Soriano, 1999). X-gal staining occurred in the testis in *Hspa2*-cre;ROSA26 males (Fig. 2b), but not in wild type littermates (Fig. 2a). Enzymatic activity was present in spermatocytes in day 20 animals and by day 30 was present additionally in spermatids, but not in spermatogonia at the periphery of the seminiferous tubule, consistent with previous reports of *Hspa2* expression (Rosario *et al*, 1992; Dix *et al*, 1996a). Expression of *Hspa2*-cre was detected in the brain of some transgenic animals by X-gal staining (Fig. 2f), but not in the brain of wild type animals (Fig. 2e), consistent with RT-PCR results. Endogenous HSPA2 expression in brain of juvenile animals (Figs. 2g and 2h) was detected by immunohistochemistry. HSPA2 was observed primarily within the ependymal cells of the lateral ventricles, confirming other recent findings with an *Hspa2*-GFP transgene (unpublished observations). Transgene expression was not detected in heart, muscle, lung or liver (data not shown). Enzymatic activity was detected by X-gal staining in the spleen (not shown) and kidneys in both *Hspa2*-cre;ROSA26 (Fig. 2d) and wild type mice (Fig. 2c). However, *Hspa2*-cre transcripts were not detected in these tissues by RT-PCR, indicating that the staining was due to endogenous β -galactosidase.

To determine more precisely when cre recombinase expression occurs during spermatogenesis and the recombination efficiency, *Hspa2*-cre males were mated to *Tg(CAGBgeo/GFP)21Lbe/J* (Z/EG) reporter females that express enhanced green fluorescent protein (EGFP) after cre-mediated excision (Novak *et al*, 2000). Cre expression was determined on sections of testes from juvenile and adult males whose genotype indicated they inherited both the cre-expressing and reporter expressing transgenes (*Hspa2*-cre;Z/EG) by immunohistochemistry with an antibody to GFP. GFP was expressed in all offspring with the *Hspa2*-cre transgene and the Z/EG reporter, indicating a recombination efficiency of 100% (n=13). At postnatal day 14, GFP immunostaining was detected during the first wave of spermatogenesis within some seminiferous tubules of transgenic mice (Fig. 3a). Non-specific staining in the interstitium was caused by the second antibody (data not shown). Using an antibody to HSPA2, it was determined that GFP expression was coincident with HSPA2 expression in leptotene/zygotene spermatocytes (Fig. 3b). The images shown are from sections of the same testis. Neither GFP nor HSPA2 were detected in spermatogonia at the periphery of the seminiferous tubules and GFP was not detected in any germ cells in wild type animals (data not shown).

At postnatal day 20, GFP was detected in pachytene spermatocytes (Fig. 3c). The localization of cre expression during the meiotic phase of spermatogenesis in *Hspa2*-cre;Z/EG males and not in spermatogonia at the periphery of the seminiferous tubule, again coincided with the pattern of HSPA2 expression (Fig. 3d). These results confirmed that the *Hspa2*-cre transgene was transcribed and translated similarly to the endogenous *Hspa2* gene during spermatogenesis.

Upon mating *Hspa2*-cre transgenic mice to mice with a floxed *Cdc2a* allele, we discovered recombination-mediated excision events were not restricted to spermatocytes. Excision of the floxed gene was detected by PCR analysis of tail biopsies of offspring resulting from matings of *Cdc2a* mice with one wild-type allele and one floxed allele (*Cdc2a*^{+/floxed}) with *Hspa2*-cre male or female transgenic mice (data not shown). This generated offspring with one wild-type allele, one disrupted *Cdc2a* allele and the *Hspa2*-cre transgene (*Cdc2a*^{+/-}; *Hspa2*-cre). Excision of the floxed allele occasionally was incomplete, allowing the detection of all three *Cdc2a* allele forms (*Cdc2a*^{+/floxed/-}; *Hspa2*-cre) within somatic tissues. In most cases, the excised allele appeared predominant over the floxed allele. RT-PCR analyses determined that *Hspa2*-cre expression did not occur at detectable levels in somatic tissues or ovary (Fig. 1c), suggesting excision of the floxed allele was a result of transgene expression during embryogenesis. This was confirmed by analysis of Mendelian ratios of the genotypes of offspring resulting from mating studies. When *Cdc2a*^{+/floxed/-}; *Hspa2*-cre male mice were crossed with *Cdc2a*^{floxed/floxed} females, 25% of offspring were expected to be *Cdc2a*^{floxed/-}; *Hspa2*-cre mice, but only 7.8% were of this genotype (Table 1). Similar ratios were observed when female *Cdc2a*^{+/-}; *Hspa2*-cre mice were mated with *Cdc2a*^{floxed/floxed} males (data not shown). These results demonstrate *Hspa2*-cre transgene expression occurs during embryogenesis and confirm a previous report that CDC2A is required for embryonic development (Santamaría *et al*, 2007). These results also indicate that the *Hspa2*-cre transgene is either not expressed in some embryos or is expressed at a low level during embryogenesis that allows some floxed *Cdc2a* alleles to escape excision.

Expression of an EGFP transgene driven by the rat *Hspa2* (*Hst70*) gene promoter during embryogenesis was reported recently (Rupik *et al*, 2006). EGFP expression coincided with the period of organogenesis, but was noted primarily in tissues involved in the development of the nervous system. Initial characterization of the rat *Hspa2* promoter in transgenic mice indicated expression occurred similarly to HSPA2 in the mouse (Widlak *et al*, 1995), but later studies reported that expression occurred in a wider variety of tissues including the

ovary, oviduct and uterus (Scieglińska *et al*, 1997). While the rat promoter might be regulated differently in the mouse or the expression pattern seen might be the result of ectopic expression, this suggests a closer examination of *Hspa2* expression during mouse embryogenesis is warranted.

Although expression of the *Hspa2*-cre transgene during embryonic development limits the usefulness of these mice for studying cell cycle genes essential for both embryonic development and meiosis, they represent a useful tool for defining the roles of genes expressed at different times during spermatogenesis or expressed in spermatogenic cells and in other tissues in the adult.

MATERIALS AND METHODS

Generation of *Hspa2*-cre Transgenic Mice

A 907-bp promoter fragment, corresponding to the mouse *Hspa2* genomic sequence-932/-25 relative to the start codon, (Dix *et al*, 1996b) was amplified from 129 SvEv genomic DNA using primers designed with a *SpeI* recognition site at the 5' end of the forward primer (5'-CCGACTAGTAGGAAAGCCGAGGGAGAAAGTT) and an *XhoI* recognition site on the 5' end of the reverse primer (5'-TACCTCGAGAACGTTAGGACGAAAGCGTCAG). The resulting product was cloned into the pBS185 plasmid 5' of the cre recombinase coding sequence, replacing the hCMV promoter. The *Hspa2*-cre fragment was then amplified and subcloned into pBluescript II KS(+) (Stratagene, La Jolla, CA) using engineered *HindIII* and *PstI* sites. A 3' bovine growth hormone (BGH) polyadenylation sequence was added to the 3' end. In an attempt to reduce positional effects on expression of the transgene, the 53-bp *Acrv1* insulator sequence (Reddi *et al*, 2003) was amplified from the CMVmin-91 Luc plasmid and placed 5' of the *Hspa2* promoter and 3' of the BGH polyadenylation sequence (Fig. 1a).

A 2.8-kb fragment produced by digestion with *AhdI* and *PvuII* was purified and microinjected into pronuclei of fertilized eggs from C57BL/6 mice. Transgenic animals were produced by the NIH Transgenic Mouse Development Facility (University of Rochester, contract # NO1-DE-12634). The resulting transgenic line was named C57BL/6-*Tg(Hspa2-cre)1Eddy/J (Hspa2-cre)* and has been transferred to the Jackson Laboratory (Bar Harbor, ME) for future distribution (JAX Stock Number 008870).

To assess cre expression, transgenic mice were crossed with *Gt(ROSA)26Sor^{tm1Sor}/J* (ROSA26) and *Tg(CAG-Bgeo/GFP)21Lbe/J (Z/EG)* reporter mice. All animal studies were approved by the NIEHS Institutional Animal Care and Use Committee and carried out according to U.S. Public Health Service (USPHS) guidelines.

Identification of Founder Animals

Transgenic founders were identified by PCR screening of genomic DNA isolated from tail biopsies using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA) with primer pairs IHGI 5089F (5'-TTGAAGCTACCCCCTAACACACTA)/cre 1487R (5'-TTGCCCTGTTTCACTATC); and cre 484F (5'-AATGTCCAATTTACTGACCGT)/IHGI 2148R (5'-TTGAAGCTACCCCCTAACACACTA). Southern analysis was used to confirm transgene integration. Briefly, genomic DNA isolated from the testis was digested with *HindIII* and *SpeI*, separated on a 1.0% agarose (w/v) gel, and transferred to Hybond-N nylon membrane (GE Healthcare-Life Science, Piscataway, NJ). A 1003-bp probe, corresponding to the cre recombinase-coding region was amplified with primers cre 484F and cre 1487R. For routine genotyping the generic cre standard PCR protocol (version 1) from The Jackson Laboratory was used. Products were separated on 2% (w/v) agarose gels.

RT-PCR for cre mRNA Expression

To assess the level of cre transcription, total RNA was extracted from brain, heart, liver, kidney, spleen, testis and ovary using Trizol Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. Total RNA (1 µg) was treated with RNase-free DNase I and reverse transcribed using oligo dT primers. A 1003-bp fragment, indicative of cre expression, was amplified with primers cre 484F and cre 1487R. *Gapdh* was amplified as a control transcript. Products were separated on 2% (w/v) agarose gels.

Histochemical Analyses

Tissues were fixed in Bouins fixative (Sigma-Aldrich, St. Louis, MO), embedded in paraffin, and sectioned at 6 µm thickness using standard procedures. Slides were deparaffinized and incubated with a GFP antibody (Abcam, Cambridge, MA) or HSPA2 antiserum (Rosario *et al*, 1992). Immunolocalization was detected using the Vectastain Elite ABC kits (Vector Laboratories, Burlingame, CA) with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), and slides were counterstained with Hematoxylin QS (Vector Laboratories). Images were recorded using an Axioplan microscope (Carl Zeiss, Thornewood, NJ,) and QImaging camera and software (QImaging, Tucson, AZ).

β-Galactosidase Histochemistry

Tissues were fixed overnight in 4% paraformaldehyde (PFA) in PBS followed by immersion in 20% sucrose in PBS for 16 hours at 4°C. Frozen sections were cut 8-10 µm in thickness, washed in PBS, immersed in rinse buffer [0.1M phosphate buffer (pH 7.4), 2mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40], and stained at 37°C overnight in buffer containing 2 mg/ml X-gal (Sigma-Aldrich), 5 mM K₃Fe(CN)₆, and 5 mM K₄Fe(CN)₆. After staining, the slides were washed in PBS, post-fixed in 4% PFA, and observed using Nomarski interference microscopy.

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Fig. 1. Testis-specific expression of cre recombinase mRNA in *Hspa2*-cre transgenic mice. (a) Schematic diagram of the *Hspa2*-cre transgenic construct. Arrows indicate orientation of the insulator sequences. The position of the cre probe is shown. (b) Southern blot analysis demonstrates transgene integration in founder line 54 (*Hspa2*-cre). Wild-type (WT) DNA was used as a control. (c) cDNA from various organs isolated from adult *Hspa2*-cre animals (upper panel) and from WT and *Hspa2*-cre animals (lower panel) were analyzed by RT-PCR using primers for cre and for *Gapdh* as an internal control. Only the testis and brain showed a cre-specific band.

Fig. 2.

Cre activity is detected in the testis and brain of juvenile and adult *Hspa2*-cre transgenic mice. Frozen sections of tissues from postnatal day 20 control (a, c, and e) and *Hspa2*-cre;ROSA26 mice were stained with X-gal (b, d and f) or with antiserum to HSPA2 (g and h). *LacZ* activity was not detected in the testis of wild type mice (a), but was detected by X-gal staining in spermatocytes of *Hspa2*-cre;ROSA26 males (b). Endogenous β -galactosidase activity was observed in the kidneys of control (c) and transgenic (d) mice. Cre expression was not detected by RT-PCR in the kidney of transgenic mice. *LacZ* activity was not observed in neuronal cells in the brain of wild type males (e), but was detected in neuronal cells in the brain of *Hspa2*-cre;ROSA26 males (f). Endogenous HSPA2 expression is seen

within neuronal cells (arrow) of the brain of postnatal day 14 (g) and in ependymal cells of adult (h) animals. Scale bars: 25 μ m.

Fig. 3. *Hspa2*-cre transgene expression is limited to spermatocytes and spermatids in the testis. Immunohistochemistry was performed on paraffin sections with an antiserum to HSPA2 to localize expression of the endogenous protein (a and c) and an antibody to GFP to localize expression of the *Hspa2*-cre transgene (b and d). GFP expression was seen on postnatal day 14 in primary spermatocytes in *Hspa2*-cre;Z/EG males (b) and coincides with the expression of HSPA2 at this age (a). On postnatal day 20, GFP was detected in primary spermatocytes and postmeiotic spermatids with the GFP antibody (d). Similar patterns of expression were observed for HSPA2 (c). Scale bars: 25 μm.