Strain Tasks

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Strain Information

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Stock Number	**008870**
Strain Name	**C57BL/6-Tg(Hspa2-cre)1Eddy/J**
Strain Name (asciiI)	**C57BL/6-Tg(Hspa2-cre)1Eddy/J**
Strain Name (marked up)	**C57BL/6-Tg(Hspa2-cre)1Eddy/J**
Strain Status	**Not Available**
Strain Status Text	
Strain Privacy	**Private**
Strain Privacy Note	
Standard supply	**[ ]**
Tier	**[ ]**
Pricing Strain Type	**[ ]**
Resource Organization	**[ ]**
Phenotype	Mice hemizygous for the Hspa2-Cre transgene are viable and fertile, with expression of Cre recombinase directed to the developing male germ cells by the mouse *Hspa2* (heat shock protein 2 [Hsp70-2]) promoter. The Hspa2-Cre sequences of the transgene are flanked by *Acrv1* (acrosomal vesicle protein 1 [SP-10]) insulator elements (which are believed to tether the *Acrv1* gene to the nuclear matrix in somatic cells [preventing transcription]). When these transgenic mice are bred with mice containing a *loxP*-flanked sequence of interest, Cre-mediated recombination is expected to result in deletion of the floxed sequences in leptotene/zygotene spermatocytes of the male offspring. The donating investigator reports that expression of Hspa2-Cre may be "leaky" and recombination of *loxP*-flanked sequences can occur in some cells in somatic tissues. These Hspa2-Cre mice may be useful to generate conditional mutations for studying mammalian spermatocyte development, gametogenesis and meiosis.
Development	The Hspa2-Cre transgene was designed with (from 5' to 3') a 53 bp mouse *Acrv1* (acrosomal vesicle protein 1 [SP-10]) insulator element, a promoter element from the mouse *Hspa2* locus (heat shock protein 2 [Hsp70-2]); containing the ATG start site and 892 bp of immediately upstream sequence), a 275 bp fragment of the *cre* gene, the bovine growth hormone 3' UTR and polyA sequence, and another 53 bp mouse *Acrv1* insulator element. This transgene was injected into C57BL/6 pronuclear stage embryos. Mutant mice (founder line 1) were bred to C57BL/6 mice. This strain was maintained by breeding Hspa2-Cre hemizygous males with wildtype (noncarrier) females for multiple generations prior to arrival at The Jackson Laboratory. Upon arrival, Hspa2-Cre transgenic mice were bred to C57BL/6J inbred mice (Stock No. 000664) for at least one generation to establish the colony.
Maintenance	When maintaining a live colony, hemizygotes may be bred with noncarrier (wildtype) siblings or C57BL/6J inbred mice.
Important Note	
Geographic Origin	
H2 Haplotype	
Appearance	
Marketing Blurb	These Hspa2-Cre mice have expression of Cre recombinase directed to the developing male germ cells by the mouse *Hspa2* (heat shock protein 2 [Hsp70-2]) promoter. The Hspa2-Cre sequences of the transgene are flanked by *Acrv1* (acrosomal vesicle protein 1 [SP-10]) insulator elements (which are believed to tether the *Acrv1* gene to the nuclear matrix in somatic cells [preventing transcription]). When these transgenic mice are bred with mice containing a *loxP*-flanked sequence of interest, Cre-mediated recombination is expected to result in deletion of the floxed sequences in leptotene/zygotene spermatocytes of the male offspring. The donating investigator reports that expression of Hspa2-Cre may be "leaky" and recombination of *loxP*-flanked sequences can occur in some cells in somatic tissues. These Hspa2-Cre mice may be useful to generate conditional mutations for studying mammalian spermatocyte development, gametogenesis and meiosis.

Synonyms
No synonyms found.

Items & Pricing
No items found.

Fees
No fees found.

Supply notes
No suppply notes found.

Licensing
No licensing found.

Strain Type
No bio strain types found.

Related alleles and markers
No alleles and markers found.

Allele-Marker Relationships
None found.

Controls

000664	C57BL/6J	- - Public

References
No references found.

Persons

Donating Investigator	Edward Mitch Eddy, NIEHS-NIH

Research Areas - Strain Related

Research Tools: Cre-lox System (Cre-Recombinase Expression: Germline/Embryonic Expression)	Public
Research Tools: Developmental Biology Research(Cre-lox System)	Public
Research Tools: Genetics Research(Mutagenesis and Transgenesis: Cre-lox System)	Public
Research Tools: Genetics Research(Tissue/Cell Markers: Cre-lox System)	Public
Research Tools: Reproductive Biology Research(Cre-lox System)	Public
Research Tools: Reproductive Biology Research(male germ cells)	Public
Research Tools: Reproductive Biology Research(testicular cell marker)	Public
Research Tools: Reproductive Biology Research(transplantation marker for embryonic and adult tissue)	Public

Research Areas - Allele Related
No allele-related research areas found.

Research Areas - Marker Related
No marker-related research areas found.

Strain OMIM Terms
No OMIM terms found.

OMIM terms for Markers related to strain
No related marker/OMIM terms found.

Strain Mammalian Phenotype Terms
No MP terms found.

Mating Scheme
No mating schemes found.

Links
No links found.

Relationships
No relationships found.

Notes

Internal Technical Note	Mary Ann Handel email with Mitch Eddy "I spoke with Leah Rae Donahue of Genetic Resources and she is willing to import the Hspa2-Cre mouse pre-publication if you are willing. As per what you and I have discussed, it would be available to me (Mary Ann Handel) right away after recovery and would be available to the general public after you and Amy publish."/// J:129028 (PMID:17932037) = Abhyankar MM, J Biol Chem 2007 282(50):36143-54 = Reddi?s Acrv1 (SP-10) insulator elements = "Regulation of cell type-specific gene transcription is central to cellular differentiation and development. During spermatogenesis, a number of testis-specific genes are expressed in a precise spatiotemporal order. How these genes remain silent in the somatic tissues is not well understood. Our previous studies using the round spermatid-specific mouse SP-10 gene, which codes for an acrosomal protein, revealed that its proximal promoter acts as an insulator and prevents expression in the somatic tissues. Here we report that the insulator tethers the SP-10 gene to the nuclear matrix in somatic tissues, sequestering the core promoter in the process, thus preventing transcription. In round spermatids where the SP-10 gene is expressed, this tethering is released." --- "3' UTR BGH is bovine growth hormone from hrGFP II-C vector (Cat# 240144 Vitality hrGFP II-C Mammalian Expression Vector).///"	added on 03-FEB-09 by JBECK
	29-jan-2009 (jbeck): Mitch Eddy essential email answers "Components of the Transgene: 5'end - 53 bp Acrv1 insulator element; promoter element from Hspa2 locus containing the ATG start site and 892 bp immediately upstream; 275 bp fragment of cre gene from pBS186 vector; 3' UTR of BGH (?) from phrGFPII-C plasmid (Stratagene); 53 bp Acrv1 insulator element ? 3' end" -- "Genetic background of embryos: Tg construct injected into C57BL/6 pronuclear stage embryos" -- "Genetic background on which strain maintained: C57BL/6 by mating hemizygous male with wild type female (also backcrossing onto 129)" -- "No dizygous mice in pedigree; will provide hemizygous mice -- Founder line: #1 -- No other incidental mutant loci are carried in this strain. -- Genomic insertion(s): location unknown -- Evidence of multiple copies (head-to-tail): not determined -- Manuscript status: Studies using this Cre mouse remain to be completed." ///	added on 03-FEB-09 by JBECK
	14-apr-2009 (jbeck): Mitch Eddy informs that "the strain can be made public as soon as they are rederived and colony expanded." and the manuscript in preparation will include the JR#. --- Eddy recommends "the Acrv1 sequence is not a primary component defining the cre expression pattern. It is an insulator element from the Acrv1 gene. The pattern of cre expression is determined by the Hspa2 promoter element. I recommend deletion of Acrv1 from the nomenclature." --- Eddy did not answer the question (((Are the 53 bp Acrv1 insulator elements you used the same as the "50-bp subfragment of the SP-10 insulator (which includes TDP-43 binding sites)" as described in PMID:17932037 (Abhyankar, Urekar, and Reddi 2007 J Biol Chem 282:36143-54)))) --- Eddy deleted the following text from original PHENO text regarding Acrv1 insulator elements "which function to tether the DNA to the nuclear matrix in somatic tissues (preventing transcription)."///	added on 16-APR-09 by JBECK
	17-apr-2009 (jbeck): jbeck asked "Are the 53 bp Acrv1 insulator elements you used the same as the "50-bp subfragment of the SP-10 insulator (which includes TDP-43 binding sites)" as described in PMID:17932037 (Abhyankar, Urekar, and Reddi 2007 J Biol Chem 282:36143-54))))" and Eddy replied "Yes, but 2 bp of genomic sequence flanking the 5' end and one bp of genomic sequence flanking the 3' end of the 50-bp insulator were included to engineer restriction enzyme sites to facilitate cloning." --- jbeck asked why they deleted the following text from original PHENO text regarding Acrv1 insulator elements "which function to tether the DNA to the nuclear matrix in somatic tissues (preventing transcription)." and Eddy replied "The evidence	added on 23-APR-09 by JBECK

that the insulator element functions to tether the DNA to the nuclear matrix in somatic tissues is indirect. It would be OK to say 'The Acrv1 insulator sequence is believed to tether the SP-10 gene to the nuclear matrix in somatic cells.' "///

Primary Colony Information

No primary colony found.

JAXStrain Version 3.6.1

JAX Stock No. 008870

from Mitch Eddy (NIH/NIEHS) [E] [eddy@niehs.nih.gov] 4-14-2009

Genotyping Protocol for Hspa2-Cre

Primers: oIMR0042 Sequence 5'- 3'- *CTAGGCCACAGAATTGAAAGATCT*
oIMR0043 Sequence 5'-3'-*GTAGGTGGAAATTCTAGCATCATCC*

Product Size: 324 bp (internal control)

Primers: oIMR1084 Sequence 5'- 3' -*GCGGTCTGGCAGTAAAAACTATC*
oIMR1085 Sequence 5'- 3'- *GTGAAACAGCATTGCTGTCACTT*

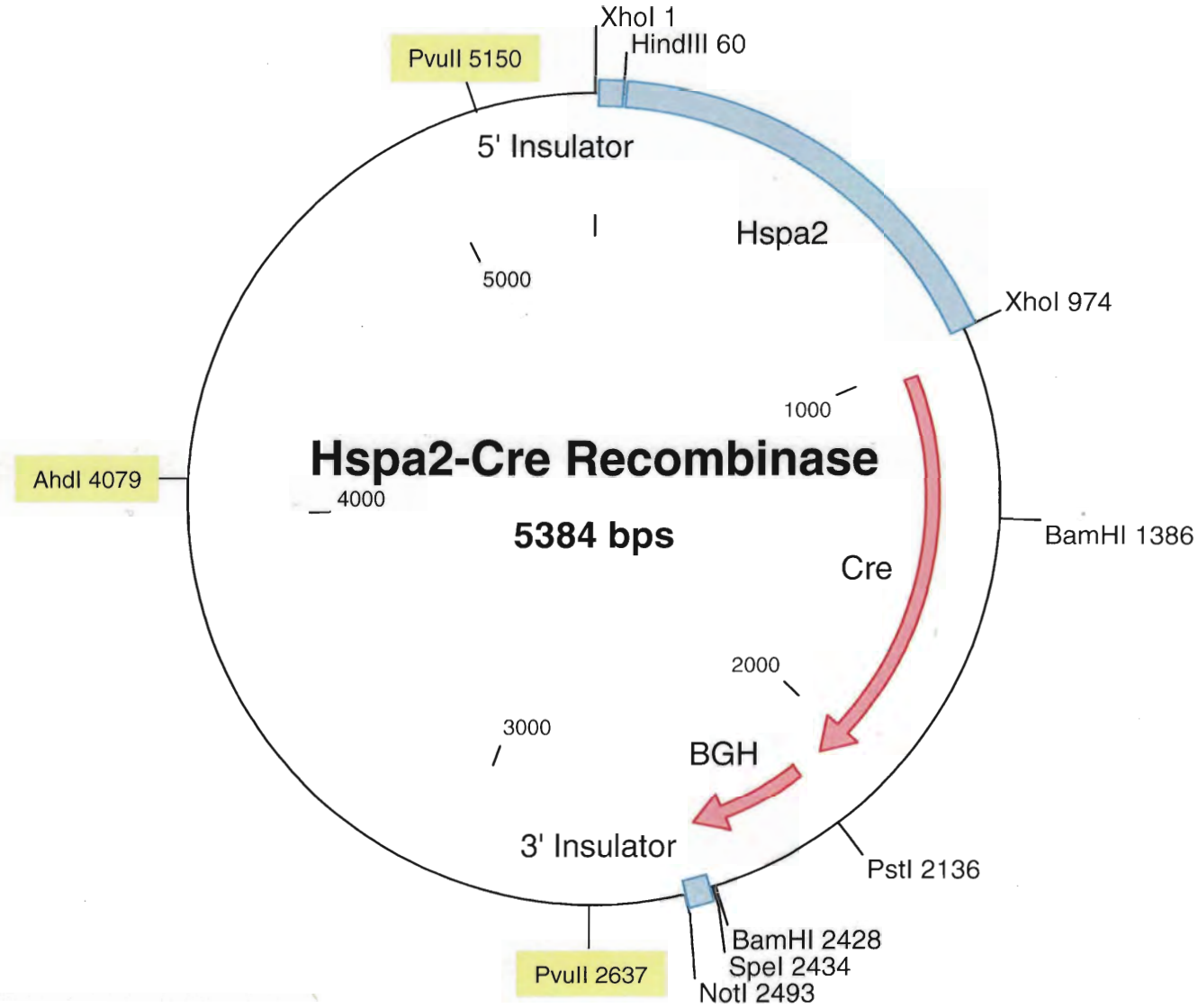
Product Size: 100 bp (transgene)

PCR master mixes were based on the following for a 20 ul reaction:

Reagents	(1x)	(15x)	**(20x)**	(30x)
PCR water	8.75 ul	131.25 ul	175.0 ul	262.5 ul
IOX AmpliTaq Buffer	2.0 ul	30.0 ul	40.0 ul	60.0 ul
2.5 mM dNTPs	2.0 ul	30.0 ul	40.0 ul	60.0 ul
Primer 1 at 10 pmol/ul	1.5 ul	22.5 ul	30.0 ul	45.0 ul
Primer 2 at 10 pmol/ul	1.5 ul	22.5 ul	30.0 ul	45.0 ul
Primer 3 at 10 pmol/ul	1.5 ul	22.5 ul	30.0 ul	45.0 ul
Primer 4 at 10 pmol/ul	1.5 ul	22.5 ul	30.0 ul	45.0 ul
AmpliTaq	0.25 ul	3.75 ul	5.0 ul	7.5 ul
	19.0 ul			
gDNA	1.0 ul			
Total	20.0 ul			

PCR, Program-using 0.2 ml tubes

94.0°C	2 minutes	
94.0°C	30 seconds	**Repeat** 35X
51.7°C	1 minute	
72.0°C	1 minute	
72.0°C	2 minutes	
4.0°C	hold	



Hspa2-Cre fragment size = 2871 bp

AhdI, PvuII double digest to release insert (band sizes = 1071 bp and 1442 bp)

Jason Beckwith

From: Eddy, Mitch (NIH/NIEHS) [E] [eddy@niehs.nih.gov]
Sent: Friday, April 17, 2009 4:13 PM
To: Jason Beckwith
Subject: RE: Hspa2-Cre mice to Induced Mutant Resource Repository--The Jackson Laboratory (008870)
Attachments: JB Response.doc

Jason,
Please see attached for responses.
Mitch

Dr. Eddy
Thank you very much for your reply. I have a few additional items.

A) In your reply, you wrote that "the Acrv1 sequence is not a primary component defining the cre expression pattern. It is an insulator element from the Acrv1 gene. The pattern of cre expression is determined by the Hspa2 promoter element. I recommend deletion of Acrv1 from the nomenclature." When these mice arrive, I will submit the transgene (and strain) nomenclature as shown below (without the Acrv1 nomen). If MGI does not accept the nomenclature, I will let you know.
JAX Stock Number 008870
C57BL/6-Tg(Hspa2-cre)1Eddy/J
(synonym: Hspa2-Cre)
OK

B) In your reply, you "strikethrough" the following question but did not answer it. Can you please clarify as yes or no (and elaborate if needed):
Acrv1 insulator elements ??
**Are the 53 bp Acrv1 insulator elements you used the same as the "50-bp subfragment of the SP-10 insulator (which includes TDP-43 binding sites)" as described in PMID:17932037 (Abhyankar, Urekar, and Reddi 2007 J Biol Chem 282:36143-54)?

Yes, but 2 bp of genomic sequence flanking the 5' end and one bp of genomic sequence flanking the 3' end of the 50-bp insulator were included to engineer restriction enzyme sites to facilitate cloning.

C) In your reply, you deleted the following about Acrv1 insulator elements "which function to tether the DNA to the nuclear matrix in somatic tissues (preventing transcription)." I anticipate our technical support group would get calls asking why these insulator sequences were included in the transgene design, so I would like to include a descriptor of the function/reasoning for using them. Is the statement incorrect or please describe their function?

The evidence that the insulator element functions to tether the DNA to the nuclear matrix in somatic tissues is indirect. It would be OK to say "The Acrv1 insulator sequence is believed to tether the SP-10 gene to the nuclear matrix in somatic cells."

D) Can you kindly let me know when the manuscript is accepted/in press?

Yes

E) Please let me know if you have any questions regarding the invoice.

The expenditure has been approved and the invoice will be processed through the NIEHS Comparative Medicine Branch purchasing office.

Jason Beckwith
JAX IMR
207-288-6798
Jason.Beckwith@jax.org

From: Eddy, Mitch (NIH/NIEHS) [E] [mailto:eddy@niehs.nih.gov]
Sent: Tuesday, April 14, 2009 12:22 PM
To: Jason Beckwith
Cc: Mary Ann Handel
Subject: RE: Hspa2-Cre mice to Induced Mutant Resource Repository--The Jackson Laboratory (008870)

Dear Jason,

I apologize for the slowness of my response. We had some personnel turn-over and it was necessary to delay this transfer. However, we are now back up to normal operations and are moving forward on getting the Hspa2-Cre mice sent to JAX. Breeding pairs were set up, offspring will be genotyped this week, and I am confident we will have males to send in the next week or so. They will of course not be sent until the appropriate information regarding animal health status has been provided and we are given the go-ahead by Ms. Norwood.

In addition, we have a short manuscript in preparation describing these mice and expect it to be published before the mice are ready for distribution by JAX. I have attached files containing my responses to your questions below, the construct diagram, the PCR genotyping protocol, and a letter from the University of Virginia indicating approval of the use of the insulator sequences. Please let me know if additional information is needed.

Regards,
Mitch Eddy

This is a reminder that I have not yet received a reply to the items (A-H) below. I have also re-attached the invoice.

Jason Beckwith
JAX IMR
207-288-6798
Jason.Beckwith@jax.org

From: Jason Beckwith
Sent: Tuesday, February 03, 2009 2:43 PM
To: 'Eddy, Mitch (NIH/NIEHS) [E]'
Subject: RE: Hspa2-Cre mice to Induced Mutant Resource Repository--The Jackson Laboratory (008870)

Dr. Eddy,
Thank you very much for your response. I have some final questions (B, C, and G) and some information below.

A) Stock Number, Nomenclature, and Requested Mice
I have assigned a strain name and stock number for these mice (shown below). While the strain name may endure some nomenclature changes over time, the stock number will NEVER change. Of note, the final nomenclature may not include the Acrv1 portion, but as it is a primary component defining the cre expression pattern, it may appear in the final accepted transgene name.

Comment [eme1]: The *Acrv1* sequence is not a primary component defining the cre expression pattern. It is an insulator element from the *Acrv1* gene. The pattern of cre expression is determined by the *Hspa2* promoter element. I recommend deletion of *Acrv1* from the nomenclature.

JAX Stock Number 008870
C57BL/6-Tg(Acrv1/Hspa2-cre)1Eddy/J
(also called Hspa2-Cre)

(B) Phenotype and Development/Genetic Background ??
I have reviewed the information you have already provided us. I then generated the texts below describing the mice you will be sending to The Jackson Laboratory. As these texts will appear on our webpage describing the mice, can you kindly review them for accuracy? Please mark any necessary changes (---offset with dashes--- for example).

****Strain Description (Phenotype) of Hspa2-Cre mice:**
Mice hemizygous for the Hspa2-Cre ~~(also called Hsp70-2 Cre)~~ transgene are viable and fertile, with expression of Cre recombinase directed to the developing male germ cells by the mouse *Hspa2* (heat shock protein 2, MGI:92643; synonym ~~or *Hsp70-2*)~~ ~~promoter and mouse *Acrv1* (acrosomal vesicle protein 1 or SP-10) insulator elements~~. The Hspa2-Cre sequences of the transgene are flanked by *Acrv1* (acrosomal vesicle protein 1, MGI:104590; synonym SP-10) insulator elements~~; which function to tether the DNA to the nuclear matrix in somatic tissues (preventing transcription)~~. When these transgenic mice are bred with mice containing a *loxP*-flanked sequence of interest, Cre-mediated recombination is expected to result in deletion of the floxed sequences in ~~the developing~~ leptotene/zygotene spermatocytes ~~and spermatids~~ of the male offspring (PMID:8631503 Dix et al., 1996, Develop. Biol. 174:310-321). These Hspa2-Cre mice may be useful to generate conditional mutations for studying mammalian spermatocyte development, gametogenesis and meiosis. The expression of Hsp2-Cre may be leaky and recombination of *loxP*-flanked sequences can occur in some cells in somatic tissues.

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****Strain Development of Hspa2-Cre mice:**

The Hspa2-Cre ~~(also called Hsp70-2 Cre)~~ transgene was designed with (from 5' to 3') a 53 bp mouse *Acrv1* (acrosomal vesicle protein 1, MGI:104590; synonym ~~or~~ SP-10) insulator element (PMID:17932037 Abhyankar et al. 2007, J. Biol. Chem. 282:36143-54), a promoter element from the mouse *Hspa2* locus (heat shock protein 2, MGI:92643; synonym ~~(or~~ *Hsp70-2*); containing the ATG start site and 892 bp of immediately upstream sequence), a 275 bp fragment of the *cre* gene, the bovine growth hormone 3' UTR and polyA sequence, and another 53 bp mouse *Acrv1* insulator element. This transgene was injected into C57BL/6 pronuclear stage embryos. Mutant mice (founder line 1) were bred to C57BL/6 mice. This strain was maintained by breeding Hspa2-Cre hemizygous males with wildtype (noncarrier) females for multiple generations prior to arrival at The Jackson Laboratory Repository.

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~~**C) Acrv1 insulator elements ??**~~

~~**Are the 53 bp Acrv1 insulator elements you used the same as the "50-bp subfragment of the SP-10 insulator (which includes TDP-43 binding sites)" as described in PMID:17932037 (Abhyankar, Urekar, and Reddi 2007 J Biol Chem 282:36143-54)?~~

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D) PCR Protocol and Construct

**Please send the Hspa2-Cre PCR protocol at your earliest convenience (MSWord or Excel file preferred).

(Please see attached)

**If readily available, please also send the sequence and/or restriction map for the Hspa2-Cre transgene. If too difficult to obtain in an efficient fashion, do not send it (although we may need it in the future for detailed genotyping if necessary).

(Please see attached)

E) Contribution to costs of importation and cryopreservation (ATTACHMENT)

I see that you had graciously offered to try to contribute $2600 to the cryopreservation and rederivation effort. Please note that your mice were accepted based on merit, and not contingent on your supplication of funds, so any funds you can provide are generously appreciated. I have attached the form for generously providing such a donation. This document may be used as an invoice or bill for services to be rendered and presented to your appropriate department to generate a purchase order. To send the funds, please follow the instructions contained therein and do not hesitate to contact me with questions regarding this issue.

(We are arranging to have the form sent.)

F) Reference to JAX Mice

It is beneficial to mention in your current or next publication using this strain that it will be available at The Jackson Laboratory with the **JAX Stock No. 008870** (and perhaps include the URL to our query page http://jaxmice.jax.org/query). This decreases the number of phone inquiries that we, and you, may receive about the strain. Please consider inserting this information into the text of your next manuscript using these mice (often the Methods section is most appropriate). Please note, this strain will appear on our public webpage upon arrival of your mice on our campus.

(Manuscript in preparation will include the above recommended information.)

G) Strain Public or Hold ??
In co-ordination with Mary-Ann Handel, we (The Jackson Laboratory Repository (JAX)) will import a single group of your Hspa2-Cre mice. This will be used to rederive the mutant mice and the resulting mice will be split between Mary-Ann Handel and JAX. As per what you and Mary-Ann Handel discussed, the Hspa2-Cre mice would be available to Mary Ann Handel right away after the strain is imported and rederived/recovered. Normally, once JAX receives mice from a donating investigator, that strain will appear on our public webpage. This may precede the publication. **Would you like these mice to be made public on our JAX Repository/JAX Mice website upon their arrival (with the typical 6 month to 1 year minimum wait until living mice are rederived and built up to be available to the public), or would you rather the strain remain private until the paper is accepted and in press?**

(The strain can be made public as soon as they are rederived and colony expanded.)

H) Importation Process Begins
**I have initiated the importation of these mice. We will request the mice listed below.

JAX Stock Number 008870: C57BL/6-Tg(Acrv1/Hspa2-cre)1Eddy/J
(also called Hspa2-Cre)
3-5 HEMIzygous males (as close to 8-12 weeks of age as possible)

-- THESE ARE IDEAL NUMBERS, but we can certainly accept less if needed -- If you are unable to produce the requested mice or it will be very difficult/costly, please let me know and I may be able to alter the request.

(We can provide 3-5 males hemizygous for the transgene. They may be slightly younger than 8 weeks when shipped.)

-- We will use our in-house stocks of C57BL/6 females to rederive the mutant mice in our vivarium.

OK

The person you assigned as the Shipping Contact should soon be contacted by our Importation Specialist (usually Kathy.Norwood@jax.org). They will provide the shipping instructions and co-ordinate a shipping date with you.

Because of the many strains we import, our importation facility is currently operating at near capacity. If you have mice ready and have not been contacted with shipping details in the next 7-10 days, please let me know.

I look forward to your reply.

Jason Beckwith
JAX IMR
207-288-6798
Jason.Beckwith@jax.org

Because of the many strains we import, our importation facility is currently operating at near capacity. If you have mice ready and have not been contacted with shipping details in the next 7-10 days, please let me know.

I look forward to your reply.

Jason Beckwith
JAX IMR
207-288-6798
Jason.Beckwith@jax.org

From: Eddy, Mitch (NIH/NIEHS) [E] [mailto:eddy@niehs.nih.gov]
Sent: Thursday, January 29, 2009 3:18 PM
To: Jason Beckwith
Subject: RE: Hspa2-Cre mice to Induced Mutant Resource Repository--The Jackson Laboratory

Dear Jason,

I think I have jumped over the last hurdle and can now move forward with transferring Hspa2-Cre mice to The Jackson Laboratory-Induced Mutant Resource Repository. The hang-up has been that the transgene contains insulator elements that were obtained from Dr. Prabu Reddi at the University of Virginia under an MTA. However, that issue has been resolved (see PDF attached).

I will have to go through channels here to get approval to send mice to The Jackson Laboratory. I will begin that process immediately, but before I can put through my request, I need to know how many, what age and what sex mice will be needed. We have a few hemizygous male (2) and female (5) mice born in November and December that could be used for breeding. In addition, we have 3 hemizygous males with September birthdates that could be used for breeding or IVF.

I also have attached a partial list of answers to your questions below. I am waiting for answers from NIEHS Comparative Medicine Branch for questions 3 and 5, but am sending the available answers now to see if you need any other additional information. I also will need to get a Material Transfer Agreement and Animal Transfer Agreement signed by the Technology Transfer Officer at The Jackson Laboratory and approved by the NIEHS Technology Transfer Agent and will do that in parallel with other arrangements.

Thank you for your patience and I will look forward to hearing from you again.

Regards,

Mitch Eddy

From: Jason Beckwith [mailto:Jason.Beckwith@jax.org]
Sent: Thursday, December 11, 2008 9:55 AM
To: Eddy, Mitch (NIH/NIEHS) [E]
Subject: RE: Hspa2-Cre mice to Induced Mutant Resource Repository--The Jackson Laboratory

This is a friendly reminder that I have not yet received your reply to these questions. Once I receive this information, I can proceed with the importation of these mice. Please see below.

Thank you very much. I look forward to your reply.

Jason Beckwith
JAX IMR
207-288-6798
jason.beckwith@jax.org

From: Jason Beckwith
Sent: Tuesday, October 21, 2008 1:49 PM
To: 'eddy@niehs.nih.gov'
Subject: Hspa2-Cre mice to Induced Mutant Resource Repository--The Jackson Laboratory

Dr. Eddy,

As part of a co-importation agreement with Mary Ann Handel, The Genetic Resources Committee will import your **Hspa2-Cre** mice for inclusion in The Jackson Laboratory-Induced Mutant Resource Repository.

I would like to initiate the process of importing your strain(s) to the Induced Mutant Resource. The information requested below is a required step in this process prior to importing any strain. The information is necessary for The Jackson Laboratory (JAX) colony managers to set up optimal breeding conditions, so please try to address each point as accurately and completely as possible.

If anything is unclear, do not hesitate to contact me. In some instances, additional emails or phone calls may be necessary.

Thank You
Jason Beckwith

Item 1) DEVELOPMENT/GENETIC BACKGROUND AND PHENOTYPE

* For the components of the transgene, please describe the individual elements and their origin (for example, promoter fragment is from Hspa2 locus containing the ATG start codon and 2.5kb immediately upstream, cre gene from p25cre vector)...please provide references if needed.
Mitch Eddy (1-29-09): Components of the Transgene: 5'end - 53 bp Acrv1 insulator element; promoter element from Hspa2 locus containing the ATG start site and 892 bp immediately upstream; 275 bp fragment of cre gene from pBS186 vector; 3' UTR of BGH (?) from phrGFPII-C plasmid (Stratagene); 53 bp Acrv1 insulator element – 3' end

* How and what genetic background was your transgenic strain generated on (example; Tg injected into FVB/N fertilized embryos)?
Mitch Eddy (1-29-09): Genetic background of embryos: Tg construct injected into C57BL/6 pronuclear stage embryos

* If different than described above, what genetic background is the strain maintained on now? Please specify the substrain used (if known).
* Have you maintained these mice by backcrossing transgenic mice to an inbred strain each generation (if so which inbred strain)--or-- by breeding transgenic mice to wildtype siblings each generation --or—by breeding transgenic mice together --or-- or other?
* After backcrossing, have you ever attempted to make this strain HOMozygous? Are HOMozygotes viable?
Mitch Eddy (1-29-09): Genetic background on which strain maintained: C57BL/6 by mating hemizygous male with wild type female (also backcrossing onto 129)

* Do you plan to provide us with HOMozygous or HEMIzygous mice?
Mitch Eddy (1-29-09): No dizygous mice in pedigree; will provide hemizygous mice

* If you are currently backcrossing (or have completed backcrossing) your strain, what is the generation (N) number? That is, how many generations have you been backcrossing for?
jbeckwith: N/A

* If you are backcrossing -- At any point in the backcross, have you backcrossed a female heterozygote to a wildtype male (thus fixing the Y chromosome to the backcrossed background)?
jbeckwith: N/A

* Usually, a unique number/letter is assigned by a laboratory to each stably transmitted insertion, generating a unique founder line designation. This assignment is usually done at the time germline transmission is confirmed. The number can have some intra-laboratory meaning or simply be a number in a series of transgenes produced by the laboratory. Since no specific line # was designated in the publication, I can simply use the default founder line 1. If you would like to specify a founder line number, please let me know.
Mitch Eddy (1-29-09): Founder line: #1

* Often, Cre-lox strains are crossed to other mutant strains. Ideally, a pure Cre strain would maintained separate from those mice bred to other mutant strains. Apart from the **Hspa2-Cre** transgene, are any incidental mutant loci (i.e. cre, lacZ, neo, loxP, frt) present in this strain (as a remnant of crossing these mice to other mutant strains)? If yes, please explain.
Mitch Eddy (1-29-09): No other incidental mutant loci are carried in this strain.

* Often, Cre-lox strains are crossed to other mutant strains. Ideally, a pure Cre strain would maintained separate from those mice bred to other mutant strains. Do these **Hspa2-Cre** mice contain, or are they likely to contain, incidental genetic background contributions (as a remnant of crossing these mice to other mutant strains)? If yes, please explain.
jbeckwith: not answered

* Do you know the genomic insertion(s) of the transgene? If yes, please explain?
Mitch Eddy (1-29-09): Genomic insertion(s): location unknown

* Is there any evidence of multiple copies (usually head-to-tail tandems) of the transgene at the insertion point? If yes, please explain.
Mitch Eddy (1-29-09): Evidence of multiple copies (head-to-tail): not determined

* Can you kindly send me a notification of when the manuscript is in press?
Mitch Eddy (1-29-09): Manuscript status: Studies using this Cre mouse remain to be completed.

* Normally, once we receive mice from a donating investigator, that strain will appear on our public webpage. This may precede the publication. If this is undesirable, please let me know (for example, I can make notes to keep the strain private until the publication appears in press).
jbeckwith: not answered

Item 2) GENOTYPING

* How do you genotype?
Mitch Eddy (1-29-09): PCR Protocol: to be sent by email

* Has a PCR protocol been developed?
Mitch Eddy (1-29-09): PCR Protocol: to be sent by email

* Does it distinguish HOMozygotes from HEMIzygotes?
Mitch Eddy (1-29-09): Does not distinguish dizygous from hemizygous

* Would you **please email the protocol to me** (MSWord, pdf, or Excel file preferred)?
Mitch Eddy (1-29-09): PCR Protocol: to be sent by email

* If called upon, could you provide the sequence and/or restriction map for the construct?
Mitch Eddy (1-29-09): Sequence and/or restriction map of construct: can be provided if needed.

* If there are additional questions regarding the protocol, who may we ask?
Mitch Eddy
LRDT, C4-01
NIH, NIEHS
111 T.W. Alexander Drive
Research Triangle Park, NC 27709
Phone: 919 541-3015; Fax 919 41-3800
Email eddy@niehs.nih.gov

Item 3) LEGAL INFORMATION (contact tjlca@jax.org)

Mouse strains in the Jackson repository are distributed for research use with the restriction that they cannot be redistributed or bred for sale to other parties. These Conditions of Use are part of our General Terms and Conditions of Sale: http://jaxmice.jax.org/cou/index.html

Some institutions have standing agreements with Jackson that cover any new strains. If your institution has a standing agreement with Jackson we will add this strain to the agreement, otherwise it will be distributed under our General Terms and Conditions.

If you have any questions or concerns or if we need to speak to your technology transfer office please contact:
Contracts Administration Office
tjlca@jax.org; 207-288-6470

Mitch Eddy (1-29-09): Does NIEHS have standing agreements with Jackson that cover any new strains? (Probably)

Item 4) SHIPPING INFORMATION and CONTACTS

* For these mice, who will be the individual that will actually be putting the mice in the shipping container? This is the person who will be contacted by our shipping specialist to arrange a delivery date.
Jacqueline Locklear, DVM
Comparative Medicine Branch
NIH, NIEHS
111. T.W. Alexander Drive
Research Triangle Park, NC 27709
Phone: 919 541-7630; Fax 919-315-4554
Email locklear@niehs.nih.gov

Item 5) Contribution to costs of importation and cryopreservation:

The current estimated costs for importation and cryopreservation are approximately $2600. Your mice were accepted based on merit, and not contingent on your supplication of funds. However, we are charged by our board of directors to ask all donating investigators if they can graciously assist this effort. Any amount is greatly appreciated. If you can contribute, I will follow up with instructions to do so.

* How much (if any) might you able to contribute towards these costs?

Mitch Eddy (1-29-09): ~$2600

Item 6) What's next? When do I ship mice?

Once I receive your response to the above questions, I can initiate the importation process for these mice. After that, you will be contacted by our Importation Specialist (Kathy.Norwood@jax.org). She will provide the shipping instructions and co-ordinate a shipping date with you.

Thank you for your attention. I look forward to your response.

Jason Beckwith
Induced Mutant Resource Repository at The Jackson Laboratory
610 Main Street Bar Harbor ME 04609
p. 207-288-6798 f. 207-288-6995
jason.beckwith@jax.org

The Jackson Laboratory Invoice No: JAXIMR008870
NIH, NIEHS PO Number: _____

Date: 3-February-2009

Bill To / Services For: Edward Mitch Eddy
P.O. Box 12233
Mail Drop C4-01
Research Triangle Park, North Carolina 27709
919-541-3015
eddy@niehs.nih.gov

Strain Name: C57BL/6-Tg(Acrv1/Hspa2-cre)1Eddy/J
(Hspa2-Cre)

JAX Contact: Jason Beckwith (Box 613)
Induced Mutant Resources Repository
The Jackson Laboratory,
610 Main Street, Bar Harbor ME 04609
207-288-6798
jason.beckwith@jax.org

JAXIMR008870 Billing Summary

Date:
3-February-2009

JAX Services Provided for Dr. Edward Mitch Eddy:
Rederivation and Cryopreservation of JAX Stock No. 008870

NIH, NIEHS PO Number:
_____

Amount Due:
2600.00 USD

Induced Mutant Resource Strain Costs (in USD)

==========

Process Cost	Approx. Cost Per Strain	Number of Strains	Approved Cost	Due
Importation and Cryopreservation	$2600	1	$2600	**$2600**

Description of Service Billed:
Induced Mutant Resource program costs of strain cryopreservation / rederivation.
To ensure the fastest progress of this service, this bill should be paid before the mice arrive or as soon as possible.

Please make check payable in USD to: The Jackson Laboratory

Memo note: JAXIMR008870
Stock No. 008870

Mail check to: Jason Beckwith (Box 613)
The Jackson Laboratory
610 Main Street
Bar Harbor, ME 04609

If Wiring US Dollars: Bank of America
ABA# 026-009-593
Account #: 9429239533
SWIFT: BOFAUS3N

If ACH Transfers: Bank of America
ABA# 011-200-365

Jason Beckwith

From: Steve Rockwood
Sent: Monday, October 20, 2008 9:15 PM
To: Mitch Eddy
Cc: Mary Ann Handel; Jason Beckwith
Subject: RE: Hspa2- Cre

Hello Mitch,

Thanks so much for agreeing to allow us to distribute your Hspa2-Cre mice. I'm going to ask one of our scientific curators to start collecting some technical information from you. Did you want to field those questions yourself or would you rather we deal with a lab manager or perhaps post-doc? Do send along a first draft of the manuscript as soon as it's in reasonable shape. Of course, we'll treat it strictly confidentially—we routinely receive pre-published materials. At some point before final publication, we'll provide you with a Jax catalog number to include in the text. That usually works well for everyone involved.

Best,
Steve

Stephen Rockwood
Manager, Strain Acquisition
The Jackson Laboratory
610 Main Street
Bar Harbor, Maine, USA
Tel: 207-288-6437
Fax: 207-288-6723

From: Leah Rae Donahue
Sent: Monday, October 20, 2008 12:23 PM
To: Mary Ann Handel; Mitch Eddy
Cc: Steve Rockwood
Subject: Re: Hspa2- Cre

Mary Ann and Mitch – We're happy to have this mouse strain come to JAX. I've forwarded your email to Steve Rockwood, our acquisitions person who will be in touch with you, Mitch. You're in very good hands! Thanks for sending your mouse to JAX. Leah Rae

On 10/17/08 4:05 PM, "Mary Ann Handel" <MaryAnn.Handel@jax.org> wrote:

Mitch – Good news. I spoke with Leah Rae Donahue of Genetic Resources and she is willing to import the Hspa2-Cre mouse pre-publication if you are willing. As per what you and I have discussed, it

would be available to me right away after recovery and would be available to the general public after you and Amy publish. (and, as I mentioned – we can't imagine having our CKO mice or results on them until considerably after you publish!)

Steve Rockwood of Genetic Resources will be contacting you. GR/Steve will handle all the paperwork (MTA, etc), which is a great relief to me – they do it all the time! I think it is through Steve that the arrangements will be made to send mice, sperm, whatever.

Thanks! Have a good weekend - m.a.

PS I voted yesterday!

On 10/16/08 12:01 PM, "Mitch Eddy" <eddy@niehs.nih.gov> wrote:

Mary Ann,

OK. I will look forward to hearing back about the mice. Sending NIH owned mice to you will require execution of a Material Transfer Agreement. I will send you an electronic versions that you will need to get signed by your institutional technology transfer person.

Keep your wallets well hidden in Prague. I had mine lifted there a couple of years ago (getting on the train to Berlin). There also was an attempt to snatch Debbie's bag containing her computer, but they ran when I yelled at them.

Mitch

From: Mary Ann Handel [mailto:MaryAnn.Handel@jax.org]
Sent: Thursday, October 16, 2008 11:46 AM
To: Eddy, Mitch (NIH/NIEHS) [E]
Subject: Re: Hsp70-2 Cre

Mitch - DON'T SAY IT YET! About becoming a blue state, that is! Just work hard and vote! I am so damned superstitious about this election and feel that every little lift of hope I get from polls, etc., will jinx it! but if you do want to feel a bit better, go here: http://election.princeton.edu/

Thanks very much for your willingness to share the Hsp70-2/Cre (old habits die slow; yes, I know – Hspa2!). I will contact Genetic Resources and get back to you. I am sure they will want this, and I know that will be a big relief to you. However, you are correct, they may require a peer-reviewed pub first. But I can find out how I can import it to best facilitate GR taking it subsequently (directly from me). So as soon as I get back to you, I think we can count on starting the process for me to import it, even if GR doesn't do it yet. I expect you are planning on keeping it on the shelf there for a while anyway? And ... do you have frozen sperm? To be honest, I don't know what the situation is here with respect to derivation via that route – we can definitely do it, but they may want the sperm frozen in the Jax new & fancy cocktail (which really does lead to better recovery), and it may speed up

importation.

Looking forward to getting the ms from Chris. FYI, I will be out of the country Oct 30 – Nov 8, with little or no email access - IMGS in Prague! Steve and Mark will be along, so it will be a fun family trip. (and yes – I will vote before I leave!)

m.a.

On 10/16/08 11:27 AM, "Mitch Eddy" <eddy@niehs.nih.gov> wrote:
Hi Mary Ann,

Amy is still in the data collection mode, but the results continue to look good and I hope that at least a first draft will be ready by the time they head off to Arkansas.

Amy would be happy for others to benefit from using the Hspa2/Cre mice. Also, I would be interested in turning them over to the JL so we do not have to deal with shipping them to other investigators. Do you have a feeling for how much trouble would be involved in providing mice to you and to the JL at the same time? I would only try to do this if did not slow down the process of getting the mice to you. I realize there is an evaluation process before mice are accepted by the JL and they might not be interested in these mice until after a publication appears.

I received the next version of the repro32 manuscript on Friday and have been working through it again. I currently am weeding out repetition of results and excessive speculation in the Discussion and hope to have something to send to you next week.

Autumn has been pleasant here as well. Even the political climate has been reasonable and there is hope we might be a blue state on Nov 4.

Cheers, Mitch

From: Mary Ann Handel [mailto:MaryAnn.Handel@jax.org]
Sent: Thursday, October 16, 2008 7:40 AM
To: Eddy, Mitch (NIH/NIEHS) [E]
Subject: Hsp70-2 Cre

Hi Mitch – how is Amy coming with respect to her publication? Although I am not sure you said so explicitly when we met several weeks ago, I am under the impression that the KO was made using the Hsp70-2 Cre that you and Amy developed. Would you consider distributing the Cre mice (or frozen sperm) prior to publication? We really have a need for that Cre for a conditional KO of a protein that localizes to the XY body (the only Cre's that we have available don't have such optimal timing). We certainly wouldn't be in a position to have any results until after you publish - as we both know, from both ends, importation can be a frustratingly long process, not to mention breeding the right mice for the CKO. If you are willing and able, please propose any sort of collaborative agreement that you think appropriate.

Is Chris having success with the repro32 manuscript?

I hope all is well there – we are having an amazing Indian summer, no frost yet. This weekend may change that, and even so, all the leaves are falling off the trees anyway!

Best – m.a.

****

Mary Ann Handel, PhD
Senior Research Scientist
The Jackson Laboratory
600 Main Street
Bar Harbor, ME 04609
maryann.handel@jax.org
Tel: 207-288-6778
Fax: 207-288-6073

~*

Leah Rae Donahue
Director, Genetic Resource Science
The Jackson Laboratory
600 Main Street
Bar Harbor, Maine 04609
207-288-6235

Jason Beckwith

From: Jason Beckwith
Sent: Thursday, April 16, 2009 10:15 AM
To: tjlca
Subject: RE: Importation of 008870
Attachments: 8870 UVA Letter re Insulator.pdf

Dr. Eddy provided additional information "a letter from the University of Virginia indicating approval of the use of the insulator sequences."

Please see the attached document.

Jason Beckwith
JAX IMR
207-288-6798
Jason.Beckwith@jax.org

From: Jason Beckwith
Sent: Tuesday, February 03, 2009 3:56 PM
To: Jason Beckwith; Kathy Norwood; Muriel Davisson; Cat Lutz; Sian Clements; Tacy Robb; Sandra Rodick; Joy Froding; Sean Sullivan; Steve Rockwood; Don Peter Liu; Deborah Boswell; tjlca; Kevin Johnson; Jen Merriam; Todd Dehm; Alicia Valenzuela; Susan Cook; Melissa Osborne; Marie Ivey; Dawn King; Lawriston Wilson; Catrina Spruce
Subject: Importation of 008870

Hello All

Kathy (IMP)
Please complete at your earliest convenience. Once mice are rederived, they will be split between JAX IMR and Mary-Ann Handel (x6778)

Peter (LEGAL)
Mitch Eddy writes "Does NIEHS have standing agreements with Jackson that cover any new strains? (Probably)" -- "I think I have jumped over the last hurdle and can now move forward with transferring Hspa2-Cre mice to The Jackson Laboratory-Induced Mutant Resource Repository. The hang-up has been that the transgene contains insulator elements that were obtained from Dr. Prabu Reddi at the University of Virginia under an MTA. However, that issue has been resolved (see PDF attached)." -- "I also will need to get a Material Transfer Agreement and Animal Transfer Agreement signed by the Technology Transfer Officer at The Jackson Laboratory and approved by the NIEHS Technology Transfer Agent and will do that in parallel with other arrangements."

Kevin (GENO)
DI has not yet provided PCR for transgene (could use generic Cre for now I suppose). When they send it, I will send it along. Hyperlink to Supplemental Materials to appear in Repo/IMR DB in the near future.

Jason Beckwith
Induced Mutant Resource Repository--The Jackson Laboratory
610 Main Street Bar Harbor ME 04609
p. 207-288-6798 f. 207-288-6995
Jason.Beckwith@jax.org

UNIVERSITY of VIRGINIA
Office of Sponsored Programs

Dr. Mitch Eddy
Bldg. 101, Rm. C422A, MD-C4-01
NIH, NIEHS
111 T.W. Alexander Drive
Research Triangle Park, NC 27709

re: Simple Letter Agreement for Transfer of Materials
Materials: plasmid: pCMV min-91 Luc, to be used for the purpose of testing the insulator with HSP70-2 transgenic expression
Provider: University of Virginia (Reddi)
Recipient: NIEHS NIH (Eddy)

Dear Dr. Eddy:

This letter will reference the above MTA. You have requested the University of Virginia's consent to transfer Hspa2/Cre mice to The Jackson Laboratory. These mice were generated using a transgene containing a sequence amplified by PRC from a plasmid provided by the University pursuant to the MTA. It is our understanding that the plasmid Materials which are the subject of the MTA will not be transferred to The Jackson Laboratory and that the sequence amplified and used in the transgene is in public databases.

Based on the these understandings, the University hereby consents to your request. Please do not hesitate to contact me if you have any questions.

Sincerely,

Gerald J. Kane
Director of Grants and Contracts

Acknowledged And Agreed To:

Dr. Prabbakara Reddi

1001 North Emmet Street, PO Box 400195, Charlottesville, VA 22904
Phone: 434-924-4270 Fax: 434-982-3096

Jason Beckwith

From: Jason Beckwith
Sent: Tuesday, February 03, 2009 3:56 PM
To: Jason Beckwith; Kathy Norwood; Muriel Davisson; Cat Lutz; Sian Clements; Tacy Robb; Sandra Rodick; Joy Froding; Sean Sullivan; Steve Rockwood; Don Peter Liu; Deborah Boswell; tjlca; Kevin Johnson; Jen Merriam; Todd Dehm; Alicia Valenzuela; Susan Cook; Melissa Osborne; Marie Ivey; Dawn King; Lawriston Wilson; Catrina Spruce
Subject: Importation of 008870
Attachments: 8870i.doc; 8870 Hspa2-cre MTA (Desktop) 09-0129.PDF

Hello All

Kathy (IMP)
Please complete at your earliest convenience. Once mice are rederived, they will be split between JAX IMR and Mary-Ann Handel (x6778)

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Jason Beckwith
Induced Mutant Resource Repository--The Jackson Laboratory
610 Main Street Bar Harbor ME 04609
p. 207-288-6798 f. 207-288-6995
Jason.Beckwith@jax.org

UNIVERSITY of VIRGINIA
Office of Sponsored Programs

Dr. Mitch Eddy
Bldg. 101, Rm. C422A, MD-C4-01
NIH, NIEHS
111 T.W. Alexander Drive
Research Triangle Park, NC 27709

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Based on the these understandings, the University hereby consents to your request. Please do not hesitate to contact me if you have any questions.

Sincerely,

Gerald J. Kare
Director of Grants and Contracts

Acknowledged And Agreed To:

Dr. Prabbakara Reddi

1001 North Emmet Street, PO Box 400195, Charlottesville, VA 22904
Phone: 434-924-4270 Fax: 434-982-3096

Importation Request

LAM/CP File No. _____

Account Number: RR09781-15S1

To: Director, Laboratory Animal Health

Date: 3-feb-2009

From: Induced Mutant Resource

Genes	Strain	Source	Phone	FAX
008870	C57BL/6-Tg(Acrv1/Hspa2-cre)1Eddy/J	**Shipping Contact:** Jacqueline Locklear	919-541-7630	919-315-4554
		Protocol Contact, DI and PI: Edward Mitch Eddy	919-541-3015	919-541-3800

SYNONYMS:
Hspa2-cre or Hsp70-2-cre

Shipping Contact email:
locklear@niehs.nih.gov

Protocol Contact, DI and PI email:
eddy@niehs.nih.gov

Shipper Address
Comparative Medicine Branch
NIH, NIEHS
111. T.W. Alexander Drive
Research Triangle Park, NC 27709

http://www.niehs.nih.gov/research/atniehs/labs/lrdt/gamete/index.cfm

DI Address
NIH, NIEHS
111 T.W. Alexander Drive
LRDT; Mail Drop C4-01
Research Triangle Park, NC 27709

Please Order
3-5 HEMIzygous males (as close to 8-12 weeks of age as possible)

If you have any questions regarding the information above, contact Steve Rockwood x 6437, sfr@jax.org
02/03/09

Published 2009 Wiley-Liss, Inc. genesis 48:114–120 (2010)

TECHNOLOGY REPORT

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

Amy L. Inselman, Noriko Nakamura, Paula R. Brown, William D. Willis, Eugenia H. Goulding, and Edward M. Eddy*

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

Received 30 June 2009; Revised 21 October 2009; Accepted 3 November 2009

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)26-Sor^{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. genesis 48:114–120, 2010. Published 2009 Wiley-Liss, Inc.†

Key words: mice; transgene; cre recombinase; spermatogenesis; heat shock protein; conditional mutant

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 (Ando *et al.*, 2000; Bhullar *et al.*, 2001; Rasoulpour and Boekelheide, 2006; Rassoulzadegan *et al.*, 2002) considerably limits their usefulness for studying the roles of most genes during meiosis in males.

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 (Dix *et al.*, 1996a; Eddy, 1999; Rosario *et al.*, 1992). Translation occurs immediately after transcription in leptotene/zygotene spermatocytes and the HSPA2 protein is present at high levels in primary spermatocytes (Dix *et al.*, 1997; O'Brien, 1987). 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

Current address for Amy L. Inselman: Division of Personalized Nutrition and Medicine, National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR 72079.

Current address for Noriko Nakamura: Department of Pharmacology, Physiology and Toxicology, BBSC Marshall University, Huntington, WV 25755.

Amy L. Inselman and Noriko Nakamura contributed equally to this work.

*Correspondence to: Edward M. Eddy, Gamete Biology Section, Laboratory of Reproductive and Developmental Toxicology, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, NC 27709. E-mail: eddy@niehs.nih.gov

Contract grant sponsors: Intramural Research Program of the NIH, National Institute of Environmental Health Sciences

Published online 21 December 2009 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/dvg.20588

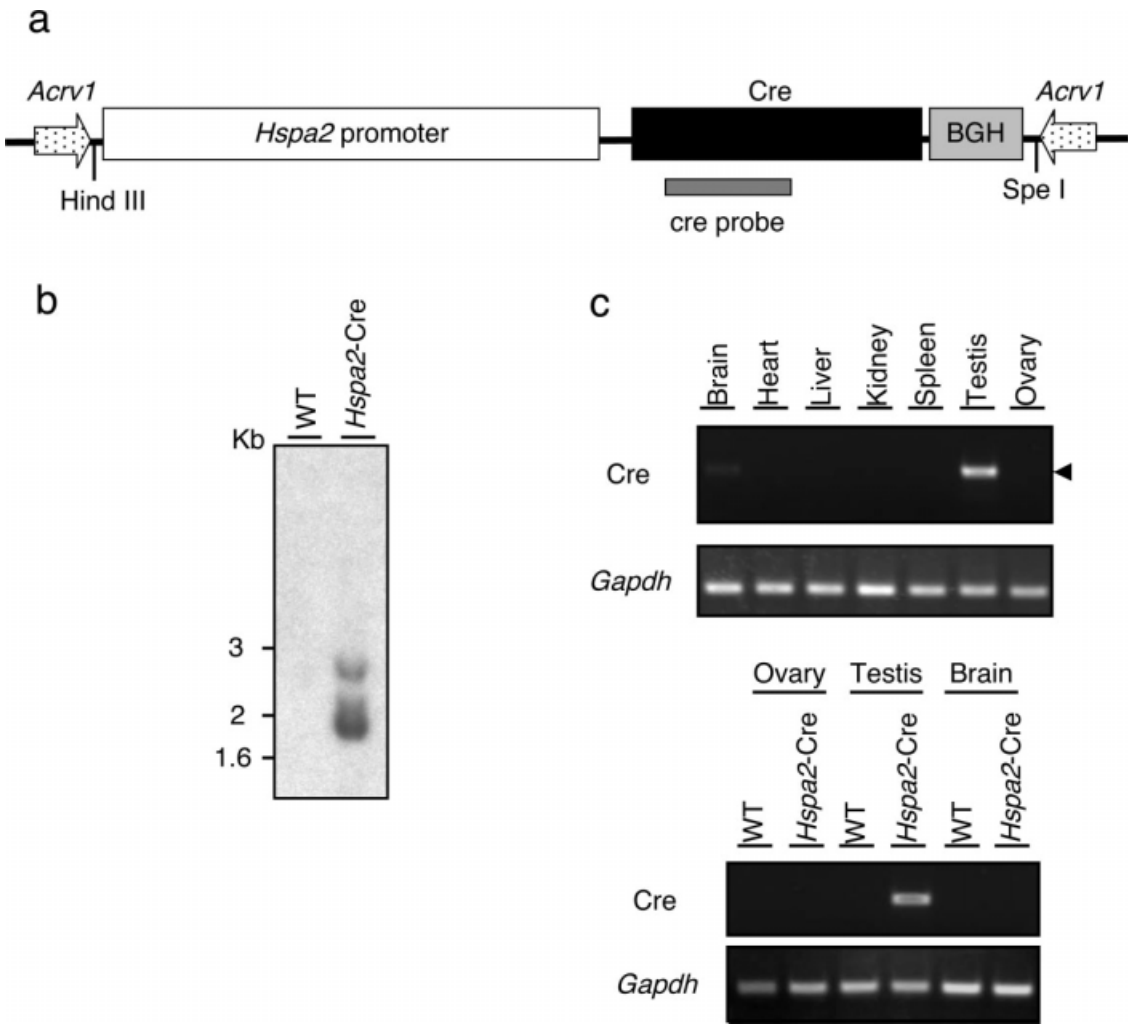

FIG. 1. Testis-specific expression of cre recombinase mRNA in *Hspa2*-cre transgenic mice. (**a**) Schematic diagram of the *Hspa2*-cre transgenic construct. Arrows indicate orientation of the insulator sequences. The position of the cre probe is shown. (**b**) Southern blot analysis demonstrates transgene 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.

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 and

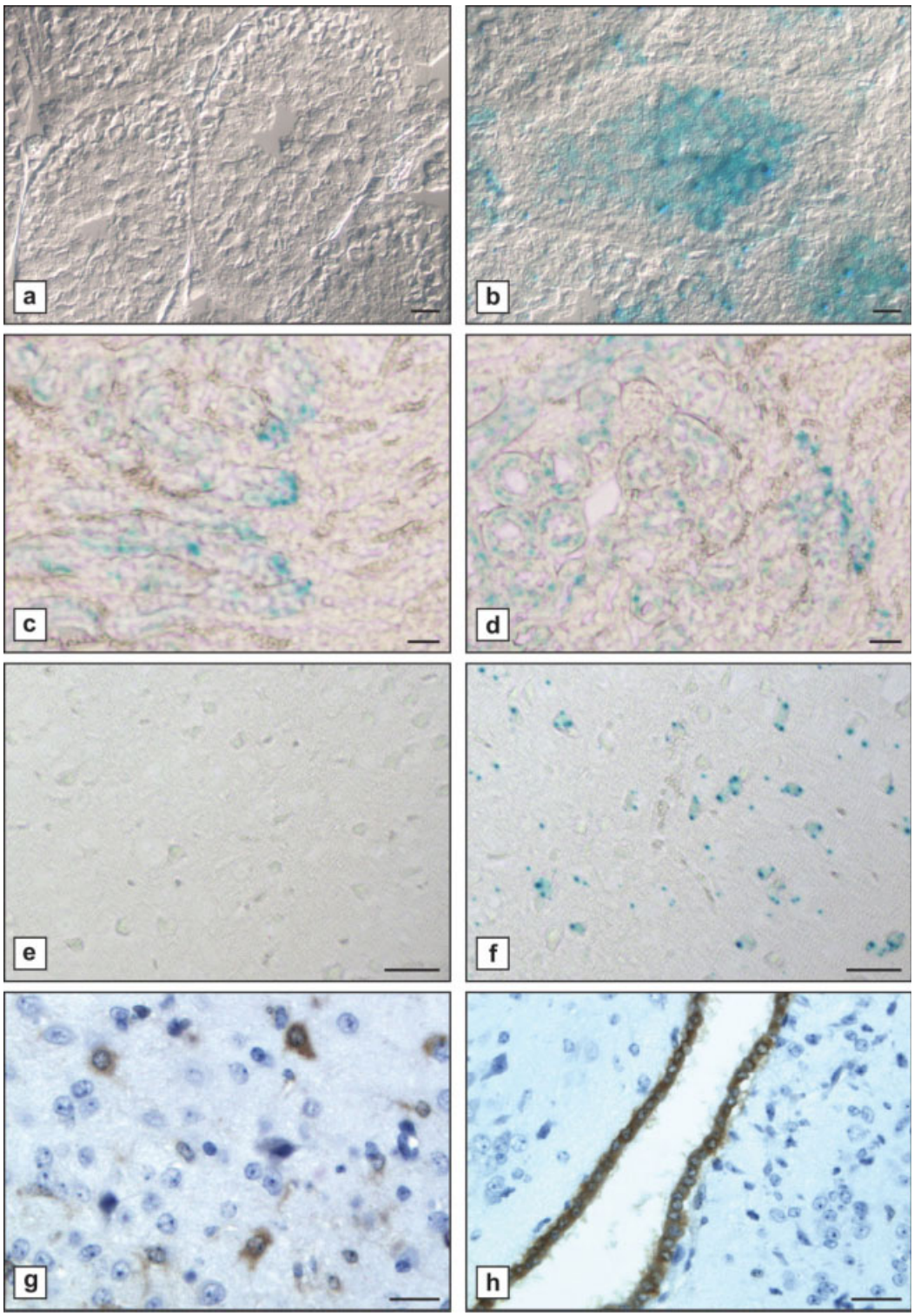

FIG. 2. Cre activity is detected in the testis and brain of juvenile and adult *Hspa2*-cre transgenic mice. Frozen sections of tissues from postnatal day 20 control (**a**, **c**, and **e**) and *Hspa2*-cre;ROSA26 mice were stained with X-gal (**b**, **d**, and **f**) or with antiserum to HSPA2 (g and 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 μm.

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 (Dix *et al*., 1996a; Rosario *et al*., 1992). 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 and adult animals (Fig. 2g,h) 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(CAG-Bgeo/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 *Hsp2a*-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. 3b). Nonspecific staining in the interstitium was caused by the secondary 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. 3a). 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. 3d). 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. 3c). 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 $Cdc2a^{+/-}$;*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 (Scieglínska *et al*., 1997). Although 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

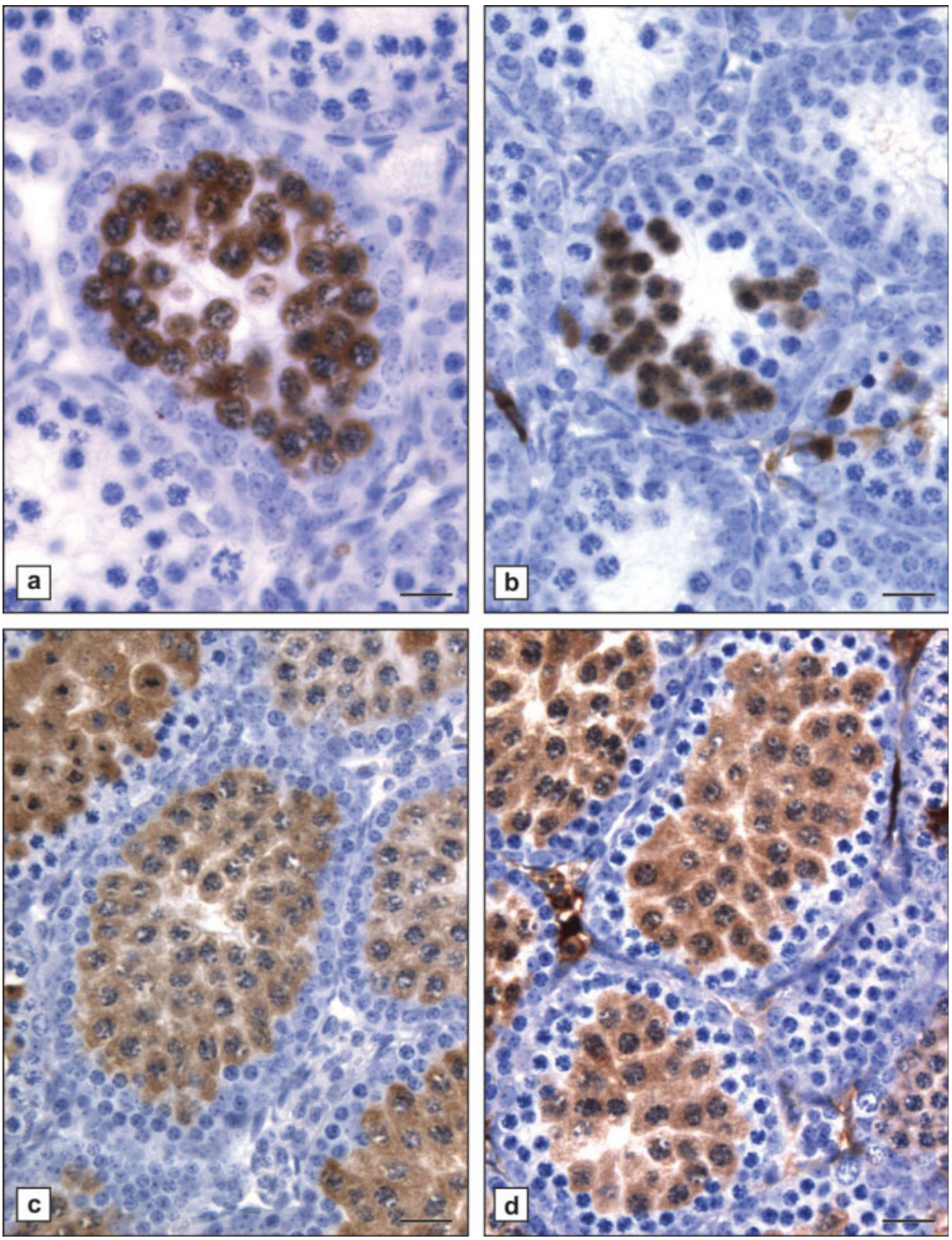

FIG. 3. *Hspa2*-cre transgene expression is limited to spermatocytes and spermatids in the testis. Immunohistochemistry was performed on paraffin sections with an antiserum to HSPA2 to localize expression of the endogenous protein (**a** and **c**) and an antibody to GFP to localize expression of the *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.

the start codon (Dix *et al*., 1996b), was amplified from 129/SvEv genomic DNA using primers designed with a *Spe*I recognition site at the 5′ end of the forward primer (5′-CCGACTAGTAGGAAAGCCGAGGGAGAAAGTT) and an *Xho*I recognition site on the 5′ end of the reverse primer (5′-TACCTCGAGAACGTTAGGACGAAAGCGT-

Table 1
Assessment of Embryonic Expression of Hspa2-cre Using a Floxed Cdc2a Targeted Line

	Observed genotype of offspring			
Breeding pair	+/f	+/−; cre	f/−	f/−; cre
Pair 65: ♂ +/−; cre ♀ f/f	15	8	13	1
Pair 68: ♂ +/−; cre ♀ f/f	8	14	6	5
Pair 75: ♂ +/−; cre ♀ f/f	1	1	0	0
Pair 76: ♂ +/−; cre ♀ f/f	2	3	0	0
% Total	33.8	33.8[a]	24.7	7.8

$n = 77$ animals.
[a]11.7% of animals with the *Cdc2a*$^{+/-}$;*Hspa2*-cre genotype showed incomplete excision by tail biopsy.

CAG). 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 *Hind*III and *Pst*I 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 *Abd*I and *Pvu*II 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 no. 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′-TTGCCCCTGTTTCACTATC); 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 *Hind*III and *Spe*I, separated on a 1.0% agarose (w/v) gel, and transferred to Hybond-N nylon membrane (GE Healthcare-Life Science, Piscataway, NJ). A 1,003-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 1,003-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 tetrahydrocholoride (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 h at 4°C. Frozen sections were cut 8–10 μm in thickness, washed in PBS, immersed in rinse buffer [0.1 M phosphate buffer (pH 7.4), 2 mM $MgCl_2$, 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 $K_3Fe(CN)_6$, and 5 mM $K_4Fe(CN)_6$. After staining, the slides were washed in PBS, post-fixed in 4% PFA, and observed using Nomarski interference microscopy.

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A Novel CpG-free Vertebrate Insulator Silences the Testis-specific *SP-10* Gene in Somatic Tissues

*ROLE FOR TDP-43 IN INSULATOR FUNCTION**

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Mayuresh M. Abhyankar[1], **Craig Urekar**[1], **and Prabhakara P. Reddi**[2]

From the Department of Pathology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Regulation of cell type-specific gene transcription is central to cellular differentiation and development. During spermatogenesis, a number of testis-specific genes are expressed in a precise spatiotemporal order. How these genes remain silent in the somatic tissues is not well understood. Our previous studies using the round spermatid-specific mouse *SP-10* gene, which codes for an acrosomal protein, revealed that its proximal promoter acts as an insulator and prevents expression in the somatic tissues. Here we report that the insulator tethers the *SP-10* gene to the nuclear matrix in somatic tissues, sequestering the core promoter in the process, thus preventing transcription. In round spermatids where the *SP-10* gene is expressed, this tethering is released. TAR DNA-binding protein of 43 kDa (TDP-43), previously shown to interact with the SP-10 insulator, was found to be in the 2 M NaCl-insoluble nuclear matrix fraction. TDP-43 prevented enhancer-promoter interactions when artificially recruited between the two by Gal4 strategy. Knockdown of TDP-43 using small interfering RNA released the enhancer-blocking effect of the SP-10 insulator in a stable cell culture model. Mutation of TDP-43 binding sites abolished this effect. Finally, a 50-bp subfragment of the SP-10 insulator, which includes TDP-43 binding sites, functioned as a minimal insulator in transgenic mice and silenced an otherwise ectopically expressed transgene in somatic tissues. The SP-10 insulator lacks CpG dinucleotides or CTCF binding sites. Thus, the present study characterized a novel vertebrate insulator in a physiological context and showed for the first time how a testis-specific gene is silenced in the somatic tissues by an insulator.

Regulation of transcription of a tissue-specific gene is central to cellular differentiation and development. Mechanisms must be in place not only to activate the gene in the correct cell type but also to keep the gene silenced in all other tissues. We study this problem using the mouse testis-specific *SP-10* gene as a model. The *SP-10* gene codes for an acrosomal protein conserved in mammals and is exclusively expressed in round spermatids (1). In this report, we address the mechanism of transcriptional silencing of the *SP-10* gene in the somatic tissues. Our previous work in transgenic mice showed that the SP-10 proximal promoter performs dual functions; it activates testis-specific transcription and also acts as an insulator preventing the possibility of transcription in the somatic tissues (2).

Insulators are DNA sequences located at gene boundaries (3). Their action prevents ectopic expression of genes they flank. Insulators are operationally defined as enhancer blockers and barrier elements. Enhancer-blocking insulators prevent a foreign enhancer from inappropriately encroaching into the promoter of the neighboring gene. The barrier insulators prevent the spread of heterochromatin and thus prevent untimely shut down of gene expression. Insulators may possess only one or both of the above properties (4).

The chicken β-globin HS4 insulator (cHS4),[3] a prototypic vertebrate insulator, possesses both of the above activities (5, 6). Binding sites for the transcription factors CTCF and USF1 have been shown to be responsible for the enhancer-blocking and barrier functions of the cHS4 insulator, respectively (7, 8). The imprinting control region located between the *Igf2* and *H19* genes and the Tsix imprinting/choice center on the mammalian X chromosome are two other well characterized vertebrate insulators with enhancer-blocking properties. The imprinting control region governs the parent of origin-specific expression of Igf2 and H19, depending on whether or not it is bound by the 11-zinc finger protein, CTCF (9, 10). Similarly, the Tsix imprinting/choice center acts as an enhancer blocker when bound by CTCF and prevents Xist gene expression, thus marking the active X chromosome (11). Both the imprinting control region and the Tsix imprinting/choice center insulators contain CpG dinucleotides, the methylation status of which dictates occupancy by CTCF. A number of other vertebrate insulators also contain CpG dinucleotides and binding sites for CTCF. These include the insulators at the ribosomal RNA genes of *Xenopus* (12), the chicken 3′-HS insulator of the β-globin gene (13), the BEAD-1 element at the human TCRα/δ locus (5), the human β-globin HS5 insulator (14), the apoB insulator of the human apolipoprotein B chromatin domain (15), and the DM1 insulator located between the human *DMPK* and *SIX5* genes (16).

In contrast, the 319-bp SP-10 insulator, which functioned as an enhancer blocker in transgenic mice, does not contain CpG dinucleotides or CTCF binding sites (2). We reported that the

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[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed: Dept. of Pathology, University of Virginia School of Medicine, P.O. Box 800904, Charlottesville, VA 22908. Tel.: 434-982-0007; Fax: 434-924-2151; E-mail: ppr5s@virginia.edu.

[3] The abbreviations used are: cHS4, chicken β-globin HS4 insulator; DBD, DNA binding domain; CMV, cytomegalovirus; TK, thymidine kinase; siRNA, small interfering RNA; TDP-43, TAR DNA-binding protein of 43 kDa.

SP-10 insulator binds to TDP-43 (TAR DNA-binding protein of 43 kDa) and that mutation of TDP-43 binding sites compromised enhancer-blocking function (17). Therefore, we anticipate that the study of the SP-10 insulator will uncover novel mechanisms of insulator function.

Here, we have addressed the mechanism of SP-10 insulator function in the endogenous context; investigated the requirement of TDP-43 for SP-10 insulator function; and, finally, determined whether a 50-bp subfragment can function as a minimal insulator capable of silencing a transgene in the somatic tissues.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Mouse spermatocyte cells (GC-2; ATCC catalog number CRL-2196) and monkey kidney cells (CV-1) were cultured in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 1% L-glutamate, and 1% nonessential amino acids. COS-7 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Flp-In CV-1 stable cell lines were purchased from Invitrogen.

In Vivo Matrix Assay—Low ionic strength matrices from cultured cells, mouse liver cells, and round spermatids were prepared essentially as described (18, 19). Following lithium diiodosalicylate-mediated extraction, the nuclei were digested with 100 μg/ml RNase-free DNase I (Roche Applied Science) for 3 h at room temperature. Matrices were collected by centrifugation for 5 min at 1500 $\times$ *g*; washed twice with extraction buffer; and resuspended in Tris-EDTA (TE), 0.1% SDS, and 1 mg/ml proteinase K. Extracts were incubated at 55 °C overnight. The DNA associated with these preparations was precipitated after phenol/chloroform extraction (matrix-bound DNA). Total genomic DNA was also extracted in parallel. PCR was performed using matrix bound as well as genomic DNA as template. The SP-10 −1.9/+1.6 kb genomic region was analyzed using primer sets, each yielding an ~270-bp amplicon at 100-bp intervals as shown in Fig. 1*A*. A typical PCR contained 10 pmol of each primer, 1 μl of 10 mM dNTP mix, 0.5 μl of *Taq*, and 5 ng of template in a 50-μl volume. The cycling conditions were 94 °C for 3 min, followed by 27 cycles of 94 °C for 30 s/55 °C for 30 s/72 °C for 30 s with a final extension at 72 °C for 5 min. The amplification was in a linear range under these conditions. Fold enrichment values were obtained by taking ratios of band intensities of amplicons generated from matrix DNA/genomic DNA. Band intensities were quantitated using ImageQuant software.

Isolation of Mouse Spermatids—Pure population (>95%) of spermatids was obtained using StaPut gradient as described previously (17, 20). Typically, testes from 10–12-week-old Swiss Webster mice were subjected to StaPut to obtain ~20–40 $\times$ 10^6 round spermatids. Alternatively, testicular germ cells from 25-day-old male mice were used as a source of round spermatids (85–90% pure). To obtain day 25 germ cells, testes from 10 mice were decapsulated and subjected to enzymatic treatment (collagenase, hyaluronidase, trypsin, and DNase) to release the cells. These were collected and washed twice with phosphate-buffered saline. Round spermatids obtained from either source were used to prepare nuclear matrix.

High Salt Matrix Preparation—High salt matrices from cultured cells were essentially prepared as described (19). In short, cells were incubated in RSB buffer for 10 min on ice, homogenized 10 times with a Dounce homogenizer by using a loose pestle, and centrifuged at 1000 $\times$ *g* for 5 min at 4 °C. Pelleted nuclei were washed twice in RSB and 0.25 M sucrose, resuspended in RSB and 2 M sucrose, and centrifuged at 34,000 $\times$ *g* for 10 min. Pelleted nuclei were washed once in RSB and 0.25 M sucrose; resuspended in RSB, 0.25 M sucrose, 1 mM $Cacl_2$ and phenylmethylsulfonyl fluoride; and digested for 3 h with 100 μg/ml RNase free DNase I (Roche Applied Science) at room temperature on a nutator mixer. After digestion, the nuclei were pelleted at 1500 $\times$ *g* for 5 min, and supernant was removed. These nuclei were then extracted with extraction buffer (20 mM Tris, pH 7.4, 10 mM EDTA) containing an increasing amount of NaCl ranging from 0.1 to 2.0 M. For each concentration of salt, the digested nuclei were washed three times with 3 pellet volumes of extraction buffer, followed by centrifugation at 1500 $\times$ *g* for 5 min. The three washes were combined at each step and mixed with loading buffer. An equal volume of each was used for Western blotting. The final 2 M salt-insoluble nuclear matrix was solubilized directly in loading buffer in an equivalent volume.

Western Blotting and Antibodies—Polyclonal antibodies to TDP-43 were raised in guinea pigs as mentioned (17). Anti-Gal4DBD (catalog number sc510) and anti-lamin A/C (catalog number sc6215) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibody to histone H3 (catalog number sc10809) was from Upstate Biotechnology, Inc. All blots were developed using an Immobilon Western kit from Millipore.

DNA Constructs—Full-length murine TDP-43 (NM_145556) was amplified from mouse testis Marathon-ready cDNA (Clontech) and cloned in pCR II-TOPO vector. This clone was used as a template to generate NH_2-terminal deletion (ΔRRM1; amino acids 169–414) or COOH-terminal deletion (ΔGly; amino acids 1–274) mutants using PCR.

Gal4 Assay Constructs—To make DNA binding domain (DBD) fusion proteins, full-length or mutant forms of TDP-43 were cloned in pFA-CMV vector (Stratagene) as BamH I-XbaI fragments in frame with the Gal4 BD. Expression of the fusion proteins in GC-2 cells was confirmed by Western blotting. The control *SP-10* open reading frame was cloned similarly. A construct expressing the p53-BD fusion was a gift from Dr. Rong Li (Dept. of Biochemistry, University of Virginia). The reporter plasmid was constructed as follows. The 5× Gal element was PCR-amplified from pFR-Luc plasmid (Stratagene) and was ligated into BglII site of pGL3 −91/+28Luc (as described in Ref. 2). Thus, the 5× Gal site separates CMV enhancer and mouse SP-10 core promoter, allowing recruitment of the Gal4DBD fusion proteins between the enhancer and the core promoter.

Luciferase Reporter Gene Constructs—Luciferase reporter constructs were built using the pGL3basic vector (Promega). Refer to Fig. 5*A* for a schematic illustration of all constructs. The coordinates for mouse SP-10 promoter sequence are as per Fig. 7 in Ref. 2 (GenBank™ accession number AF 133710). The SP-10 core promoter (−91/+28) was PCR-amplified with BglII (5′ end) and HindIII (3′ end) sites using the −408SP10-gfp

plasmid (1) as template and cloned into BglII + HindIII-cut CMV-pGL3 (2), creating construct 1 (Fig. 5*A*). The 319-bp insulator (−408/−92 portion of the SP-10 promoter) from plasmid −408SP10-gfp was PCR-amplified with BglII restriction enzyme sites on both sides and cloned into the BglII site of construct 1, creating construct 5. The 50-bp SP-10 insulator (−186/−135 of the SP-10 promoter) was PCR-amplified with BglII restriction enzyme sites from −408SP10-gfp and cloned into the BglII site of construct 1. Clones bearing the 50-bp insulator in both forward and reverse direction were generated (constructs 2 and 4 in Fig. 5*A*). Additionally, a clone containing three head to tail copies of the 50-bp insulator (*i.e.* all in forward orientation) was selected, giving rise to construct 3 of Fig. 5*A*. The 50-bp insulator bearing KpnI sites on both ends was cloned into KpnI-cleaved construct 1, which placed the insulator upstream of the CMV enhancer in the correct as well as reverse orientations, giving rise to constructs 6 and 7, respectively. To generate reporter constructs with the thymidine kinase (TK) core promoter, a 92-base pair fragment corresponding to the −61/+31 (wherein +1 corresponds to the transcriptional start site) region of the HSV TK gene promoter was PCR-amplified from pRL-TK (Promega, WI) with BglII and HindIII sites and cloned into CMV-pGL3 (2). This base construct (construct 8; Fig. 5*A*) was then used to clone in the 50-bp SP-10 insulator in both orientations as described above, creating constructs 9 and 10. All constructs were confirmed by sequencing.

Transient Transfection Assays—Transient transfections were performed in COS-7 or GC-2 cells using Mirus TransIT®-LT1 (Mirus Corp.) transfection reagent. 1.5×10^5 Cells were plated in 6-well plates, and typically, the total amount of DNA transfected per well was 1 μg. Cells were harvested 48 h post-transfection. The plasmid pRL-TK (Promega) was cotransfected at a 1:10 ratio with the reporter to normalize for transfection efficiency. Luciferase activities were measured by the Dual-Luciferase reporter assay system (Promega) according to the instructions provided with the kit. Normalized luciferase values were plotted in histograms. For Gal4DBD-based assays, 2.0×10^5 cells were seeded and transfected with 1 μg each of reporter and effector construct. The luciferase assay was done 48 h post-transfection.

Generation of Stable CV-1 Cell Lines—CV-1 stable cell lines were generated using the Flp-In system (Invitrogen) as per the manufacturer's instructions. In short, four cassettes ((i) CMV-SP10 core-luciferase, (ii) CMV-(−408/−92) insulator-SP10 core-luciferase, (iii) CMV-mutant −408/−92 insulator-SP10 core-luciferase (the two GTGTGT motifs at −172 and −160 mutated), and (iv) CMV-stuffer-SP10 core-luciferase) were initially cloned in pGL3basic vector (Promega). These cassettes were then cut out and subcloned into pCDNA5FRT vector (Invitrogen). Flp-In CV-1 cells (Invitrogen) were used to co-transfect these expression vectors with pOG44, and hygromycin-resistant clones were selected as per the manufacturer's instructions. For all clones, flippase recombinase target (FRT)-mediated site-specific integration was confirmed by Southern hybridization (data not shown).

RNA Interference—TDP-43-specific or control siRNA reagents were purchased from Dharmacon (sequences available upon request). siRNA was introduced into CV-1 cells by reverse transfection using Lipofectamine RNAiMAX (Invitrogen) as per the manufacturer's instructions. Cells were harvested 48 h post-transfection for luciferase assay.

Generation of Transgenic Mice—Transgenic mouse lines were generated using constructs 1–4 (Fig. 5*A*). Each transgene was purified as a BamHI fragment. Pronuclear injections were performed by the University of Virginia's Gene Targeting and Transgenic Facility, using standard procedures (21). Tail DNA was isolated from founder mice using the Qiagen DNeasy® tissue kit (Qiagen), and transgenic founders were identified by PCR using primers designed to amplify the entire open reading frame of the luciferase cDNA. Primers LucF (5′-GGTAAAGCCACCATGGAAGACGCCA) and LucR (5′-TTACACGGCGATCTTTCCGCCCTTC) were used as forward and reverse primers, respectively. Amplification of a 1665-bp product identified putative positive founders. The transgenic lines were subsequently confirmed by Southern hybridization using the Luciferase cDNA (Fig. 5*B*) and the CMV enhancer as probes.

Copy Number Estimation for Transgenic Mice—Southern blot hybridization was used to determine the copy number of the transgene in all of the mouse lines that were used for the analysis of luciferase expression. Ten micrograms of tail DNA was digested to completion with BglII, electrophoresed through a 1% agarose gel, and transferred to Duralon-UV™ membrane (Stratagene). Full-length luciferase coding sequence (bp 54–1746 from vector pGL3basic; Promega, WI) was radiolabeled using [α-^{32}P]dCTP and the Prime-a-Gene® labeling system (Promega). DNA hybridization was performed using ExpressHyb (Clontech) per the manufacturer's recommended protocol. The probed blot was exposed to an Amersham Biosciences storage phosphor screen and scanned in on an Amersham Biosciences Storm 860. For copy number analysis, the blot image was imported into Amersham Biosciences ImageQuant version 5.0. Within ImageQuant, a box was placed over each band on the blot, and volume quantification was performed on all bands after the subtraction of background. The Southern blot image (Fig. 5*B*) was carefully analyzed to identify bands corresponding to single copy integration *versus* multiple copy integration of the transgene. Because of the location of a BglII site at the 5′ end of the core promoter in all constructs used for making transgenic mice, single copy integration would result in a hybridization signal above 2 kb. Because of the presence of a second BglII site at the 3′ end of the CMV enhancer, head to head ligated multiple copy transgene integration would result in a strong hybridization signal at 2.4 kb for constructs 1–4. Head to tail ligated multiple copy integration would result in a hybridization signal at 4 kb for all of the constructs. The transgenic mouse line (line 540), which generated a single hybridization signal that is at neither 2.4 nor 4 kb (Fig. 5*B*, *lane 5*), represented single copy integration, and the volume of that band was taken as a standard single integration volume to determine the copy number of the rest of the lines. The volume of each hybridization band from each line was divided by that single-integration volume to determine the number of copies each band represented on the blot. The total value for each of the bands was then added up to give the total copy number of that particular mouse line. Copy number was independently estimated and confirmed using the CMV enhancer region

(XhoI-released fragment from CMV-TOPO) (2) as probe on the same blot as described above (data not shown).

Luciferase Assays on Tissues from Transgenic Mice—Tissues were harvested from 8–12-week-old transgenic male mice as well as nontransgenic littermates and immediately placed in Promega passive lysis buffer, manually homogenized, and left to sit at room temperature for 15 min. The homogenized tissues were then frozen until analysis. Upon thawing, lysed tissue samples were centrifuged at 16,000 × *g* for 2 min to remove debris; the supernatant was transferred to a new microcentrifuge tube. Soluble protein was quantified using the Bio-Rad protein quantification reagent (catalog number 500-0006) using the manufacturer's recommended protocol. The luciferase analysis was performed on a Turner Designs TD20/20 luminometer with Promega luciferase assay reagent. Luciferase assays were standardized by protein amount; 50 μg of protein was used for each tissue analyzed. The highest luciferase activity obtained from any tissue of nontransgenic littermates was 0.15. Therefore, we considered 0.15 as the background cut-off and subtracted this from the luciferase activities of transgenic mice.

RESULTS

The SP-10 Insulator Is Associated with the Nuclear Matrix—We previously demonstrated using transgenic mice as an assay system that the proximal promoter of the spermatid-specific mouse *SP-10* gene functions as an enhancer-blocking insulator in the somatic tissues. In round spermatids, where the *SP-10* gene is expressed, the insulator function is relieved. Here we investigated the mechanism of the SP-10 insulator function in its native context. Previous studies reported a close association between insulator elements and components of nuclear matrix (22–24). To determine whether the SP-10 insulator operates by tethering to the nuclear matrix, we employed an *in vivo* matrix attachment assay. In this assay, a combination of mild detergent and lithium diiodosalicylate is used to detach proteins from nuclei without disturbing the native attachment sites (18, 19). Nuclei obtained from mouse liver cells (*SP-10* gene silent) or round spermatids (*SP-10* gene expressed) were washed with mild detergent, extracted with lithium diiodosalicylate, and then digested with DNase I. After digestion, the nuclear matrices were prepared as described, and the associated DNA was purified. To determine whether the *SP-10* gene insulator is attached to the matrix, we PCR-amplified a series of 270-bp segments at 100-bp intervals spanning the −2.0 to +1.6 kb region of the mouse *SP-10* gene and plotted the -fold enrichment (Fig. 1*A*). In liver cells where the *SP-10* gene is not expressed, the region corresponding to −266/+28 was found enriched up to 20-fold in the nuclear matrix fraction as compared with the adjacent sequences on either side (Fig. 1*B*). PCR amplification using primers corresponding to the larger −408/+28 region also showed similar enrichment as the −266/+28 region (data not presented). This specific enrichment, however, was completely lost in the case of round spermatids where the *SP-10* gene is expressed (Fig. 1*B*). These results suggest that the proximal promoter region of the *SP-10* gene is sequestered within the nuclear matrix in cells that do not express *SP-10* and that it is released within the differentiating spermatogenic cells, thus allowing *SP-10* gene transcription. We confirmed this differential tethering by varying the source of the cell type. Similar to liver cells, enrichment of the SP-10 proximal promoter was observed within the nuclear matrix in GC2 tissue culture cells as well (data not shown). We used day 25 postnatal mouse testicular germ cells as an alternate source of *SP-10*-expressing cells. At postnatal day 25, a majority of the cells in mouse testis will be round spermatids because of the synchronous nature of the first wave of seminiferous epithelium. Similar to the round spermatids isolated from adult mice, the day 25 spermatids also showed no enrichment of SP-10 promoter region in the nuclear matrix (data not shown). To our knowledge, this is the first direct comparison of attachment properties of an insulator in two different scenarios of gene expression.

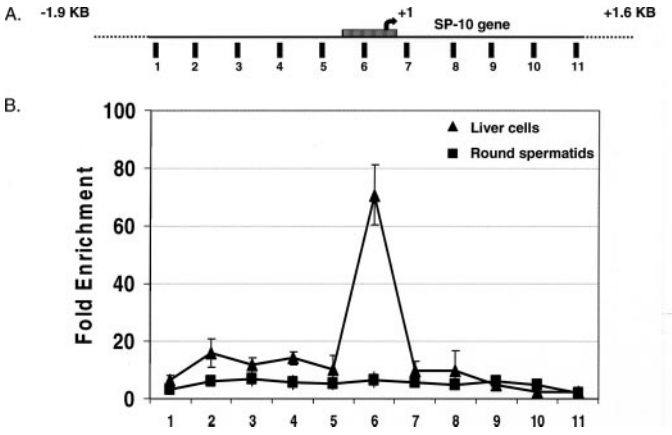


FIGURE 1. **The SP-10 insulator tethers the proximal promoter of the gene to the nuclear matrix.** *A*, schematic representation of the *SP-10* region analyzed for matrix attachment. Each *vertical bar numbered 1–11* represents an individual PCR amplicon spanning the −1.9 kb/+1.6 kb region of the SP-10 gene. The *shaded box* represents the −408/+28 region of the SP-10 promoter, +1 being the transcriptional start point. *B*, *in vivo* nuclear matrix attachment assay. Low ionic strength matrices were prepared from liver cells, and round spermatids and matrix-bound DNA fragments were extracted after extensive DNase I digestion. Enrichment of the SP-10 insulator fragment was analyzed by PCR using the primer sets depicted in *A*. Liver cell nuclear matrix showed selective enrichment of the region corresponding to the SP-10 insulator. Round spermatids showed no such enrichment. Data shown represent the average of at least three independent experiments represented as mean ± S.E. Primer sequences are available upon request.

TDP-43, the SP-10 Insulator-binding Protein, Is Co-localized in the Matrix—We previously reported identification of TDP-43 (TAR DNA-binding protein of 43 kDa) using a portion of the SP-10 insulator to screen a cDNA expression library (17). TDP-43 was originally cloned using the HIV TAR region and shown to be a transcriptional repressor (25). Buratti and Baralle (26) showed a role for TDP-43 in mRNA splicing and determined that it binds to RNA/DNA via UG/TG repeats. We previously showed that TDP-43 binds to the −186/−148 SP-10 promoter via two 5′-GTGTGT motifs located on the opposite strand. Disruption of these motifs resulted in loss of TDP-43 binding *in vitro* and compromised the stage- and cell-specific expression of a reporter gene in transgenic mice (17). These results prompted us to investigate the potential of TDP-43 as a candidate insulator protein. To begin, we first asked if TDP-43 was in the nuclear matrix. Nuclear matrix proteins are typically resistant to extraction with 2 M NaCl. We used the serial extraction method as described by Yusufzai and Felsenfeld (19). This procedure avoids miscellaneous behavior of proteins as a result of direct treatment with 2 M NaCl (27). Purified nuclei from

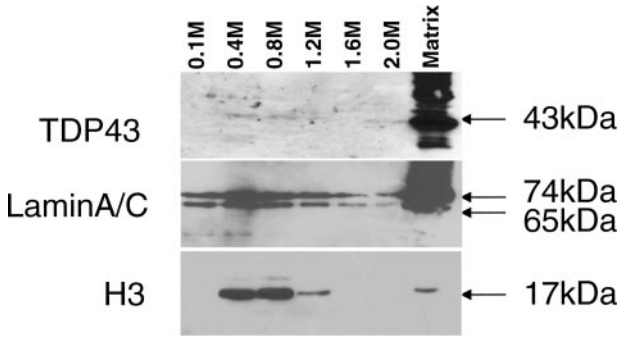


FIGURE 2. **TDP-43 is a nuclear matrix protein.** Purified nuclei from GC-2 cells were extensively digested with DNase I and serially extracted with buffers of increasing ionic strength. Release of different proteins was monitored by Western blotting. Known matrix proteins, namely nuclear lamins, were seen mainly in the matrix fraction as expected, whereas the nonmatrix protein histone H3 was extracted with lower amounts of salt. The majority of TDP-43 remained in the 2 M NaCl-resistant matrix fraction. The bands above 43 kDa could be aggregates of TDP-43.

GC-2 cells were extensively digested with DNase I and subjected to extraction with buffers of increasing ionic strength. The release of TDP-43 was monitored by Western blotting. (Fig. 2). The majority of TDP-43 was retained in the 2 M salt-resistant nuclear matrix fraction, although small amounts of the protein were extractable at lower salt concentrations. Known soluble and insoluble nuclear proteins were assayed in parallel as controls. A majority of nuclear lamins were detected in the matrix fraction as expected. In contrast, histone H3 was readily extractable with a lower amount of salt, being a nonmatrix protein. These results indicated that TDP-43 is a component of the nuclear matrix.

TDP-43 Blocks Enhancer-Promoter Interaction—In order to test the candidacy of TDP-43 as an insulator protein, we performed an enhancer-blocking assay wherein TDP-43 was artificially recruited to occupy a position between the CMV enhancer and the SP-10 core promoter. A reporter gene construct was generated in which five tandem repeats of Gal4 DNA binding sites were inserted between the CMV enhancer and the −91/+28 SP-10 core promoter (Fig. 3*A*). TDP-43 contains two RNA recognition motifs and a carboxyl-terminal glycine-rich (Gly) domain (26). We fused the Gal4 DNA binding domain to the wild type TDP-43 or its truncated versions lacking either the NH_2-terminal RRM1 domain or the COOH-terminal Gly domain (Fig. 3*A*). The Gal4 DBD fusion protein constructs were cotransfected with the above reporter plasmid into GC2 cells, and the ability of the DBD fusion proteins to block the CMV enhancer activity was measured. Luciferase activities resulting from cotransfecting DBD alone were used as 100%. Wild type full-length TDP-43 could block 60% of the enhancer activity (Fig. 3*B*). This effect was due to site-specific recruitment, because the untargeted TDP-43 did not show a similar blocking effect. Deletion of the COOH-terminal Gly domain restored the enhancer activity to near control level, indicating a role for this domain in blocking function. In contrast, the RRM1 domain appeared to play a lesser role in blocking. The SP10-DBD negative control showed no enhancer blocking, whereas the p53-DBD fusion protein, a potent transcriptional activator, elevated reporter gene activity (Fig. 3*B*). These results showed

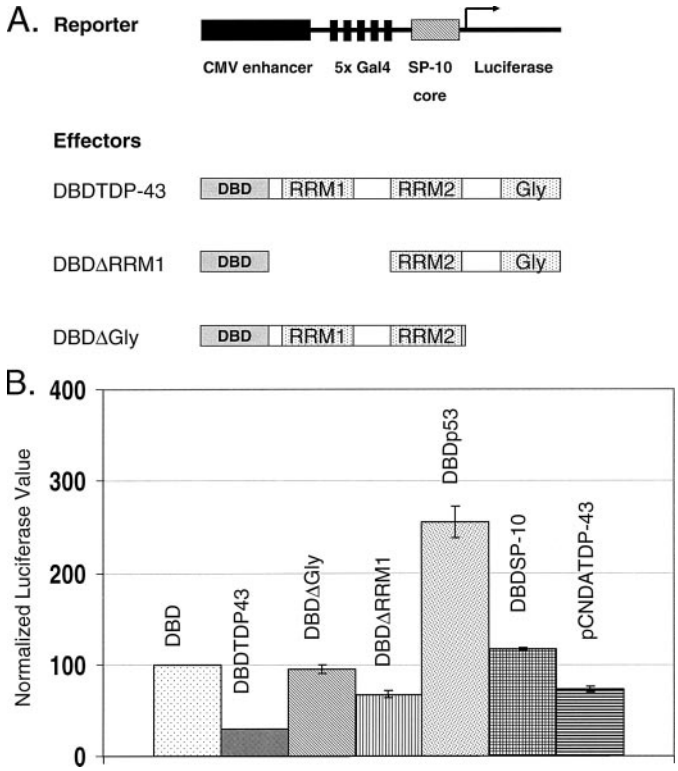


FIGURE 3. **Gal4 recruitment strategy shows that TDP-43 prevents enhancer-promoter interactions.** *A*, schematic showing the reporter and effector constructs used in an enhancer-blocking assay. The reporter plasmid allows recruitment of Gal4DBD fusion proteins between the CMV enhancer and the SP-10 core promoter. The GAL4 DNA binding domain fusion proteins used in this study include full-length TDP-43 (DBDTDP43), COOH-terminal deletion mutant (DBDΔGly), or NH_2-terminal deletion mutant (DBDΔRRM1) (not drawn to scale). Full-length p53 and SP-10 open reading frames were also generated as control DBD fusion proteins. *B*, the effector and reporter constructs were co-transfected into GC-2 cells. Cells were harvested after 48 h of transfection, and luciferase activity was measured. Luciferase values were normalized in relation to the value obtained with the DBD vector alone. pCDNATDP-43 is an untargeted form of TDP-43.

that TDP-43 could prevent the enhancer-promoter interaction and hence has a potential to act as an insulator protein *in vivo*.

Functional Link between TDP-43 and SP-10 Insulator; Knockdown of TDP-43 Released Repression of an Insulated Transgene—In order to directly test whether TDP-43 is involved in mediating SP-10 insulator function, we used the siRNA-mediated knockdown approach. To this end, first we generated a series of stable cell lines carrying in the same genomic location single copy integration of the enhancer-driven reporter gene with or without the SP-10 insulator separating an enhancer and a promoter. The constructs used to generate these lines and their luciferase activities are shown in Fig. 4*A*. The CMV enhancer plus SP-10 core promoter-driven transgene expressed a high amount of luciferase. The presence of the −408/−92 SP-10 insulator but not a stuffer fragment of similar length blocked the CMV enhancer activity. Mutation of the TDP-43 binding sites (two GTGTGT motifs at −172 and −160 mutated) compromised the enhancer-blocking ability of the SP-10 insulator. Next, to test the requirement of TDP-43 for the above enhancer-blocking function, we performed siRNA-mediated knockdown of TDP-43 in all of the above stable cell lines. To account for any off-target effects, we used a commercially available control siRNA reagent. Western blots indicated

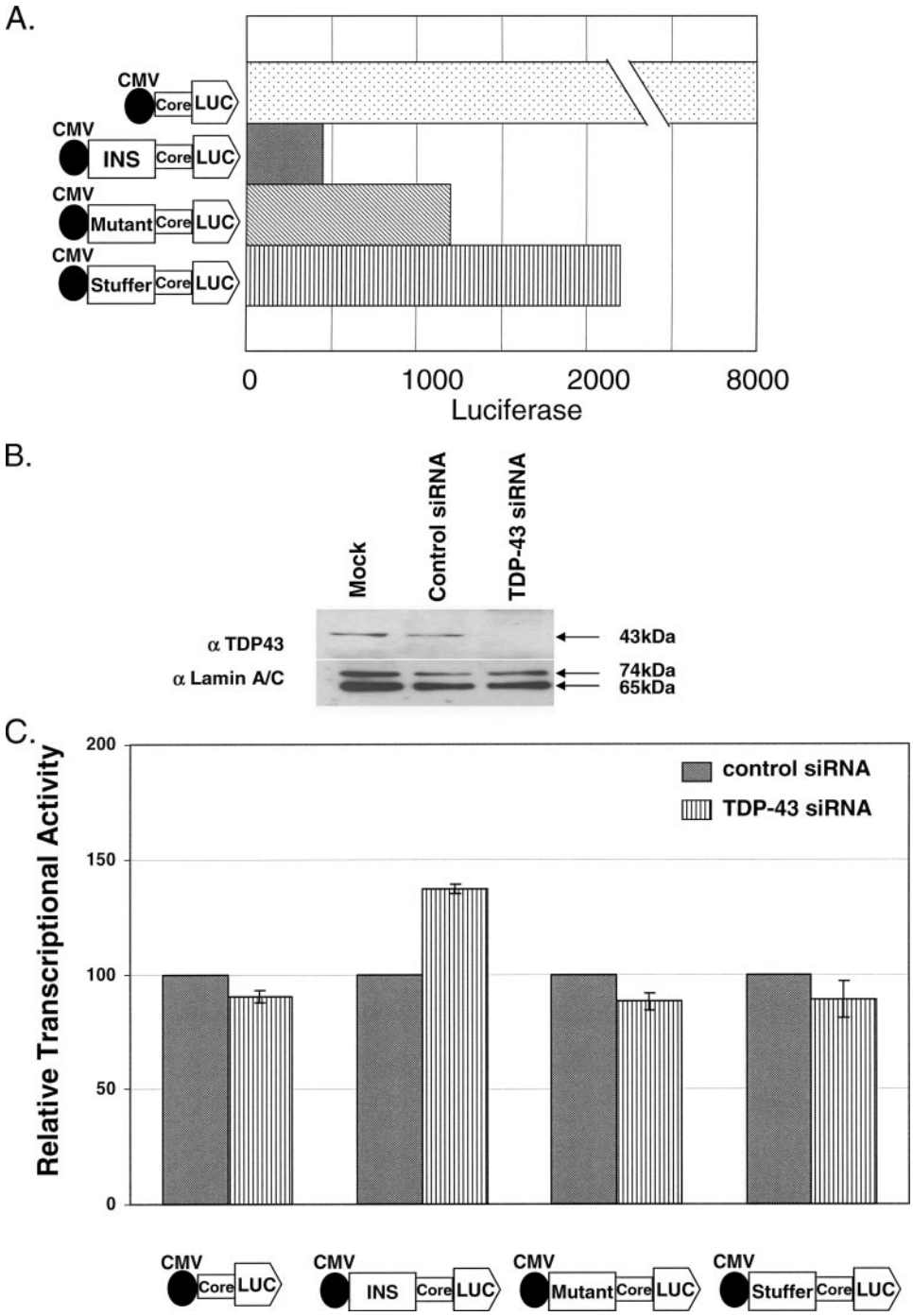
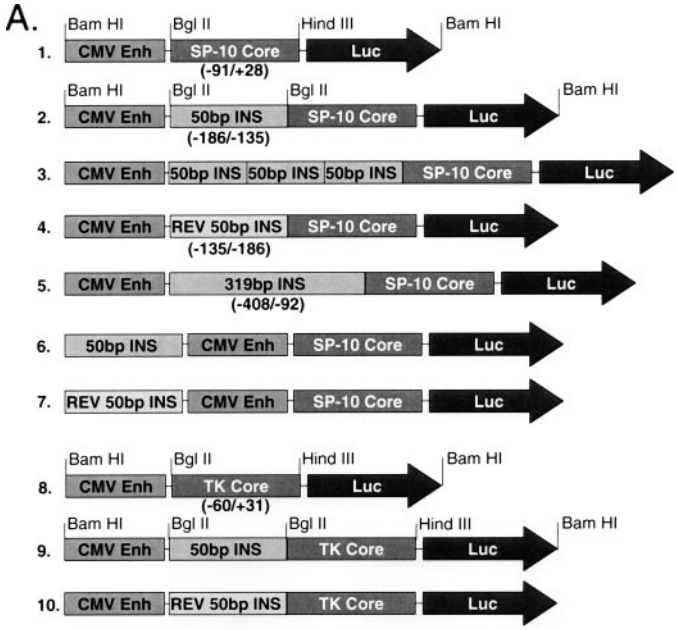


FIGURE 4. **Knockdown of TDP-43 results in release in repression of an insulated transgene in stably transfected CV-1 cells.** *A*, *graph* showing the luciferase values of CV-1 stable clones. The reporter constructs used to generate stable clones in CV-1 Flp-In cells are depicted to the *left*. The CMV enhancer plus SP-10 core promoter showed robust luciferase expression, as expected. The SP-10 insulator (*INS*) when present between the enhancer and the core promoter repressed luciferase expression by more than 5-fold, as compared with the uninsulated clone containing a stuffer fragment (*stuffer*) of identical length. Mutation of TDP-43 binding sites within the insulator (mutant) compromised the enhancer-blocking ability by 3-fold. The values represent the average of at least three independent assays. *B*, the above CV-1-stable cell lines were transfected with 10 nM control or TDP-43-specific siRNAs, as described under "Experimental Procedures." Protein extracts were prepared 48 h post-transfection. Western blots using anti-TDP-43 (*top*) or anti-lamin A/C (*bottom*) antibodies showed specific knockdown of TDP-43 protein. *C*, knockdown of TDP-43 released the enhancer-blocking effect of the SP-10 insulator. Luciferase levels in cells treated with nontargeting control siRNAs were considered as 100 for normalization. Values are shown as the means of three independent experiments ± S.E. The increase in luciferase activity of cells carrying insulated transgene is statistically significant compared with the cells lacking the insulator by Student's *t* test ($p < 0.005$). However, the luciferase activities of mutant insulator ($p > 0.1$) or stuffer ($p > 0.49$) are not statistically significant.

that TDP-43-specific siRNA completely knocked down the TDP-43 protein, whereas the control siRNA had little effect (Fig. 4*B*). For each stable cell line, luciferase values resulting from the off target siRNA were plotted as 100%. Knockdown of TDP-43 significantly elevated ($p < 0.005$) the reporter gene activity in the cells carrying the SP-10 insulator as compared with the cells containing the stuffer DNA. TDP-43 knockdown had no effect when TDP-43 binding sites in the SP-10 insulator were mutated (Fig. 4*C*). Taken together, these results indicated that TDP-43 plays a role, at least in part, in the enhancer-blocking activity of the −408/−92 SP-10 insulator.

A 50-bp Fragment of the SP-10 Insulator Functions as a Minimal Insulator—Next, we asked whether a smaller segment within the −408/−92 SP-10 insulator can function as a minimal insulator. Our previous work using progressive 5′ deletions of the −408/−92 insulator established that the −186/−92 but not the −135/−92 region acted as an enhancer blocker (2). To determine if the −186/−135 region by itself, which includes TDP-43 recognition sites, could act as a minimal insulator, we inserted the −186/−135 fragment between the potent CMV enhancer (28) and the −91/+28 SP-10 core promoter and performed enhancer-blocking assays in COS cells. The construct containing the 319-bp full-length SP-10 insulator (−408/−92) was also used for comparing the enhancer-blocking activity. Circular plasmids (see constructs 1–7 in Fig. 5*A*) were transfected into COS cells, and luciferase values were measured 48 h post-transfection. The construct containing the CMV enhancer and SP-10 core promoter expressed high levels of luciferase, whereas insertion of a single copy of the −186/−135 fragment between the enhancer and core promoter resulted in a 2.9-fold reduction in luciferase expression, indicating that the 50-bp subfragment by itself was able to block enhancer



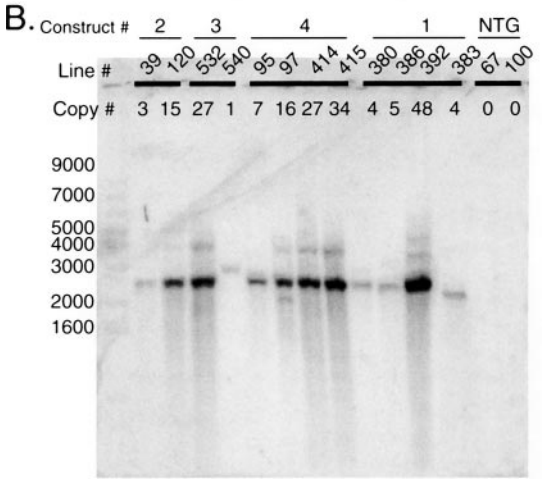


FIGURE 5. **Schematic representation of the reporter gene constructs (not drawn to scale) used in enhancer-blocking assays in tissue culture cells and in transgenic mice.** *A*, the base vector for all of the above constructs was pGL3basic (Promega, WI). The CMV enhancer (406 bp) corresponds to coordinates 59–465 bp of pEGFPN1 (Clontech). The SP-10 core promoter (−91/+28; 120 bp in size), the 50-bp insulator (−186/−135), and the 319-bp SP-10 insulator (−408/−92) fragments used in the above constructs originated from the −408SP10-gfp plasmid (16), which contains the −408/+28 SP-10 promoter (+1 corresponds to the transcriptional start site). The −60/+31 HSV-thymidine kinase core promoter (TK Core; 92 bp in size) corresponds to coordinates 668–759 bp of pRLTK vector (Promega, WI). The size of the luciferase gene is 1916 bp. Transgenic mice were generated using constructs 1–4. Although depicted in only some, BamHI sites flank all of the constructs. *CMV enh*, CMV enhancer; *SP-10 core*, SP-10 core promoter, *INS*, SP-10 insulator of indicated size; *TK core*, HSV-thymidine kinase core promoter; *Luc*, luciferase coding region and poly(A) signals. *B*, evidence for reporter gene integration in transgenic mice and determination of copy number. BglII-digested genomic DNA from all of the transgenic mouse lines were probed with radiolabeled luciferase cDNA. *Construct numbers* correspond to *A*. *Line numbers* denote independent mouse lines for each construct. Estimated copy number is indicated for each mouse line (see "Experimental Procedures" for an explanation of the size of hybridization signals and how the copy number was determined). The strong hybridization bands at the 2.4- and 2-kb region in many of the lines correspond to multiple copies of the transgene. Lack of hybridization in nontransgenic controls (*NTG*) indicates the specificity of the luciferase probe. Molecular size markers are indicated in base pairs to the *left*.

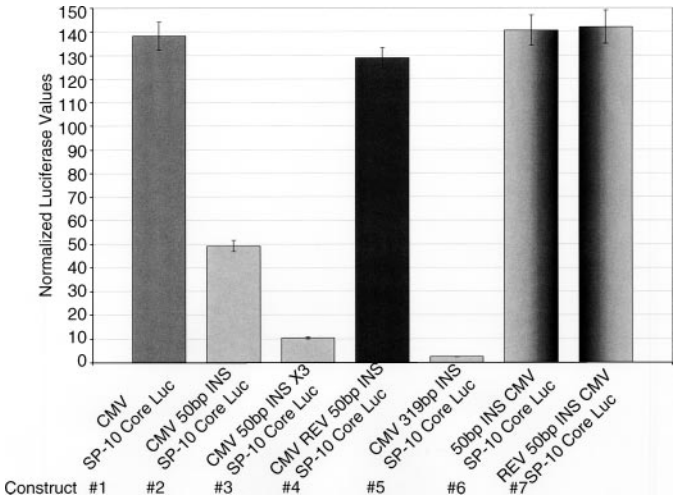


FIGURE 6. **The 50-bp (−186/−135) SP-10 insulator acts as a minimal enhancer blocker in transiently transfected cells.** Constructs 1–7 from Fig. 5*A* were used in enhancer-blocking assays. COS cells were transiently transfected with circular plasmids, and cells were harvested 48 h later. The resulting luciferase activities were normalized and plotted. Note the high CMV enhancer activity in the absence of an insulator (construct 1). The presence of one (construct 2) or three copies (construct 3) of the 50-bp SP-10 insulator between the enhancer and the core promoter reduced luciferase activity by 2.9- and 13-fold, respectively. Surprisingly, when inserted in the opposite orientation (construct 4), the 50-bp SP-10 insulator lost the ability to block the CMV enhancer. The construct containing the full-length 319 bp (−408/−92) SP-10 insulator (construct 5) was used as a positive control for enhancer blocking. When placed upstream of the CMV enhancer, the 50-bp SP-10 insulator failed to block enhancer activity, thus proving that the 50-bp fragment is a true insulator and not a transcriptional repressor (constructs 6 and 7). Normalized luciferase values obtained from six independent experiments are plotted. The mean values with S.E. are indicated.

activity (Fig. 6). A stuffer fragment containing unrelated DNA failed to inhibit CMV enhancer activity (data not shown). Enhancer blocking by the 50-bp minimal insulator was less efficient compared with that of the 319-bp full-length SP-10 insulator, suggesting that the −408/−186 region contains additional cis-elements for enhancer blocking. Three tandem copies of the 50-bp fragment, however, showed an additive effect on enhancer blocking and reduced luciferase values 13-fold. In contrast, when placed in the opposite orientation (−135/−186), the minimal insulator altogether lost its ability to block the upstream enhancer (Fig. 6). Finally, for a definitive proof of insulator function, the 50-bp SP-10 fragment was placed upstream of the CMV enhancer. A true insulator will not interfere with enhancer activity when placed upstream of the enhancer, whereas a transcriptional repressor would be expected to repress the enhancer from this location. Enhancer-blocking assays showed that the 50-bp SP-10 fragment did not block enhancer activity when placed upstream of the CMV enhancer in either orientation (Fig. 6). These data provide proof of the insulator function of the 50-bp SP-10 fragment. Taken together, the above results showed that the 50-bp (−186/−135) SP-10 fragment functions as a minimal insulator.

The 50-bp Minimal Insulator Is both Necessary and Sufficient for Enhancer Blocking—Since the 50-bp insulator originates from a testis-specific gene, we investigated if the enhancer-blocking activity of the SP-10 insulator was restricted to the

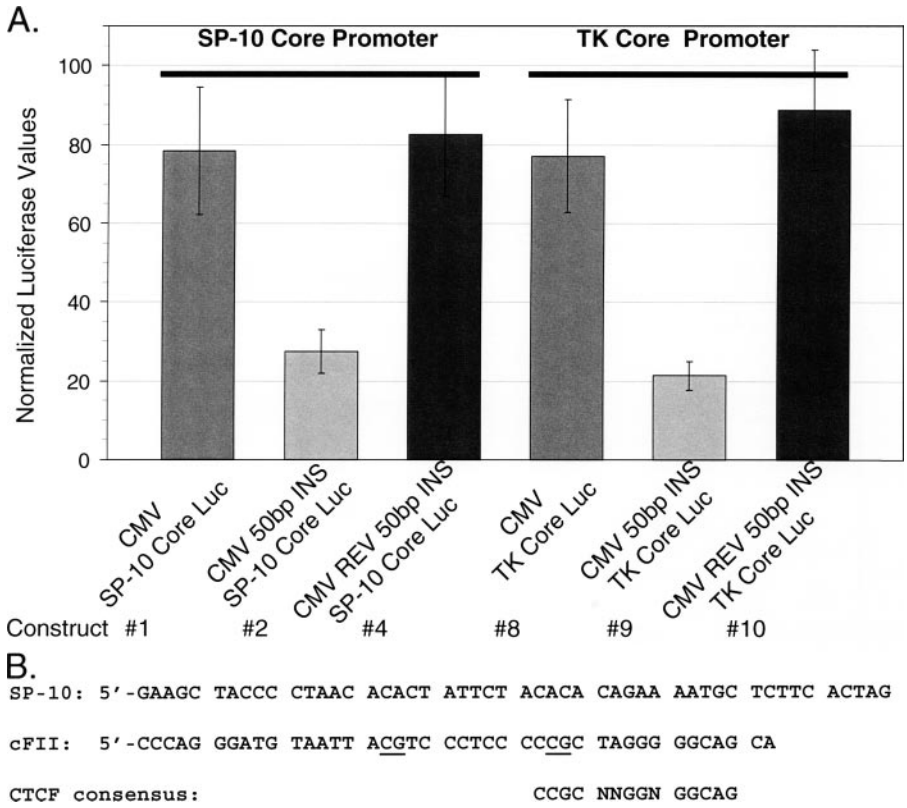


FIGURE 7. **The 50-bp SP-10 insulator is both necessary and sufficient for enhancer blocking.** *A*, Enhancer-blocking assays were performed (as in Fig. 6) in the context of the endogenous (SP-10 core) and a foreign (TK core) core promoter using the indicated constructs. The CMV enhancer activated reporter gene expression from either core promoter (constructs 1 and 8, as in Fig. 5*A*). The 50-bp SP-10 insulator blocked the CMV enhancer in the context of the unrelated TK core promoter (construct 9) to the same extent as when used with the SP-10 core promoter (construct 2). Similarly, insertion of the 50-bp insulator in the opposite orientation resulted in a loss of blocking function even in the context of TK core promoter (compare constructs 4 and 10). *B*, the 50-bp SP-10 insulator does not share sequence similarity with the cHS4 FII insulator. The nucleotide sequence of the 50-bp SP-10 insulator (SP-10) was compared with that of the 42-bp chicken globin FII insulator (*cFII*). The two mini-insulators do not share sequence similarity. Both have been shown to be sufficient for enhancer-blocking function (present study and Ref. 5). The chicken globin FII insulator is 64% GC-rich and contains two CpG dinucleotides (*underlined*). In contrast, the SP-10 insulator is A-rich. Unlike the chicken globin FII insulator, the SP-10 insulator does not contain a consensus CTCF binding site (*CTCF*) or CpG dinucleotides.

context of the SP-10 core promoter elements only. To test this, we replaced the −91/+28 SP-10 core promoter in constructs 1, 2, and 4 of Fig. 5*A* with the heterologous −60/+31 TK core promoter (constructs 8–10; Fig. 5*A*). Nearly identical enhancer-blocking activities were observed in the context of either core promoter, suggesting that the mechanism of enhancer blocking by the SP-10 minimal insulator is not limited to the context of the native SP-10 core promoter (Fig. 7*A*). Consistent with the notion of a generic mechanism, the reverse orientation of the 50-bp SP-10 insulator failed to block enhancer activity in the context of the TK promoter as well (construct 10; Figs. 5*A* and 7*A*). Thus, the above experiment using the TK core promoter proved that the 50-bp minimal SP-10 insulator is both necessary and sufficient for enhancer-blocking function.

A comparison of the 50-bp SP-10 minimal insulator and the chicken β-globin cHS4 insulator 42-bp FII portion (7) showed no sequence similarities. FII contains the canonical consensus site for CTCF, as do other well characterized vertebrate insulators, but the nucleotide sequence of the 50-bp SP-10 insulator does not bear any resemblance to the consensus CTCF site (Fig. 7*B*). The FII insulator is GC-rich and contains two CpG dinucleotides, whereas the 50-bp SP-10 insulator is devoid of CpG dinucleotides. In fact, the 50-bp SP-10 insulator is A-rich (36% of nucleotides are A; FII contains 19% A) (Fig. 7*B*).

Test for Insulator Function in a Physiological Context—The above enhancer-blocking assays were performed using circular plasmids and transient transfection assays in COS cells. Although there is evidence that plasmids do chromatinize following transfection, the higher order structure of chromatin may be considerably different *in vivo*. To test for enhancer-blocking function in native chromatin and to evaluate the potential of the 50-bp insulator in preventing ectopic expression of the testis-specific *SP-10* gene, we generated transgenic mice. The experimental paradigm consisted of first generating transgenic mice in which a luciferase reporter transgene was placed under the control of CMV enhancer and the SP-10 core promoter (construct 1; Fig. 5*A*). This was meant to mimic a situation wherein a foreign enhancer gains direct access to the SP-10 core promoter *in vivo*. Ubiquitous expression of the transgene was expected in these uninsulated mice because of the panactive nature of the CMV enhancer (29). A second set of transgenic mice harboring the 50-bp SP-10 insulator (either one or three copies) inserted between the CMV enhancer and the SP-10 core promoter (constructs 2 and 3; Fig. 5*A*) would then test whether the insulator can prevent ectopic expression caused by the CMV enhancer in a physiological context. The presence of the transgene and its copy number in independent mouse lines were determined by Southern hybridization as described under "Experimental Procedures" (Fig. 5*B*).

Transgenic mice bearing the potent CMV enhancer upstream of the SP-10 core promoter expressed the luciferase reporter gene in a number of tissues, including brain, heart, liver, kidney, spleen, muscle, epididymis, seminal vesicle, and testis (Fig. 8), indicating that the SP-10 core promoter was in fact vulnerable to activation by an adjacently located foreign enhancer. Multiple-tissue expression of luciferase varied among the four separate transgenic lines. Among somatic tissues, the highest level of expression was observed in heart, skeletal muscle, and seminal vesicle of two independent lines (383 and 386 in Fig. 8). Lines 380 and 392 expressed lower levels of luciferase in somatic tissues compared with testis (Fig. 8). High expression in heart and muscle is in agreement with previous

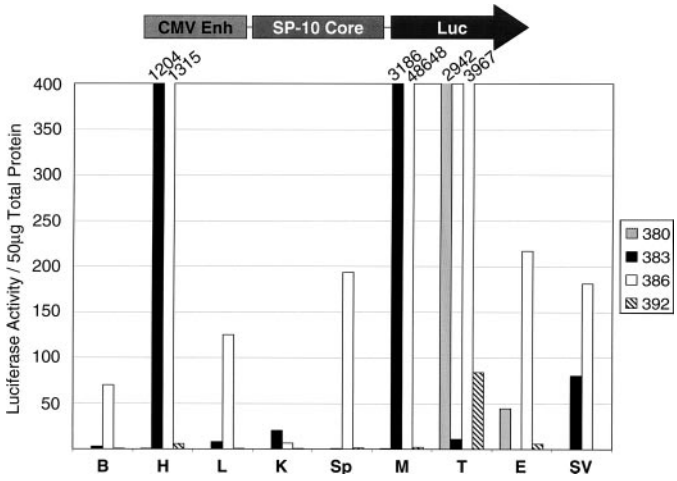


FIGURE 8. **Ectopic expression in transgenic mice due to the presence of the CMV enhancer upstream of the SP-10 core promoter.** Using the transgene shown schematically, four separate lines of transgenic mice (380, 383, 386, and 392) were generated, and their tissues were analyzed for luciferase gene expression. Multiple tissue expression was found in all four lines, indicating the panactive nature of the CMV enhancer. High expression in heart, skeletal muscle, and testis is in agreement with previous studies using the CMV enhancer in transgenic mice. Luciferase activities above background are plotted. *B*, brain; *H*, heart; *L*, liver; *K*, kidney; *Sp*, spleen; *M*, thigh muscle; *T*, testis; *E*, epididymis; *SV*, seminal vesicle.

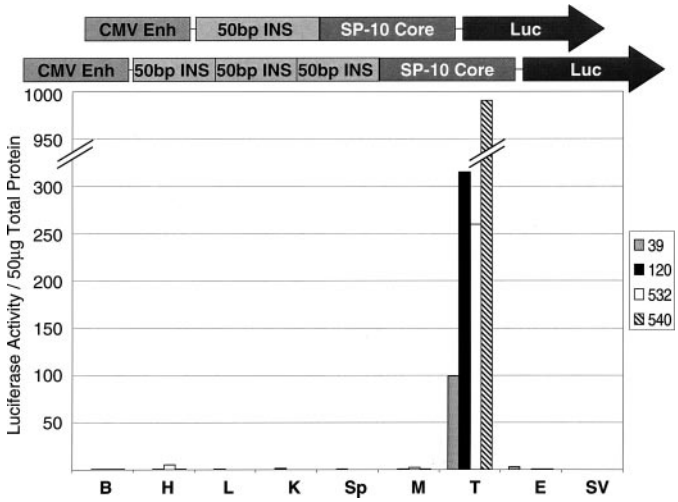


FIGURE 9. **The 50-bp SP-10 insulator blocked CMV enhancer activity in the somatic tissues but not in testis of transgenic mice.** Two lines of mice (39 and 120) bearing one copy and two additional lines (532 and 540) bearing three tandem copies of the 50-bp SP-10 insulator were analyzed for luciferase expression. In contrast to the uninsulated mice in Fig. 8, the presence of the 50-bp insulator between the CMV enhancer and the core promoter prevented expression in somatic tissues, indicating that the 50-bp SP-10 insulator blocked the CMV enhancer activity. All four lines, however, expressed luciferase in testis, indicating that the insulator does not function as an enhancer blocker in testis, where the endogenous mouse *SP-10* gene is expressed. Luciferase activities above background are plotted. *B*, brain; *H*, heart; *L*, liver; *K*, kidney; *Sp*, spleen; *M*, thigh muscle; *T*, testis; *E*, epididymis; *SV*, seminal vesicle.

reports using the CMV enhancer in conjunction with a heterologous promoter (29–32). Three of the four mouse lines expressed high amount of luciferase in testis. There was no correlation between the copy number of the transgene and the level of expression in testis or somatic tissues, suggesting that the site of integration of the transgene may have contributed to the variation in reporter gene expression. Line 392, for example, which bears an estimated 48 copies of the transgene, expressed poorly compared with the remaining three lines, each carrying 4–5 copies of the transgene (Fig. 5*B*). Overall, the results presented in Fig. 8 demonstrated that the CMV enhancer can cause ectopic expression of SP-10 core promoter-driven transgene. This, in turn, allowed us to test whether the 50-bp SP-10 insulator has the potential to prevent ectopic expression of the transgene.

The 50-bp SP-10 Insulator Blocks CMV Enhancer Activity in Somatic Tissues but Not in Testis of Transgenic Mice—In contrast to mice bearing the uninsulated transgene, none of the transgenic mice bearing the 50-bp SP-10 minimal insulator between the CMV enhancer and the SP-10 core promoter expressed luciferase in somatic tissues (Fig. 9). Two lines bearing one copy of the 50-bp insulator and two additional lines bearing three copies of the insulator were analyzed. None of the mouse lines expressed luciferase in the somatic tissues above background levels. The lack of somatic tissue expression in these insulated mice suggested that the 50-bp insulator effectively blocked CMV enhancer activity in somatic tissues (Fig. 9). It is noteworthy that a mere 50-bp insulator was able to block a panactive enhancer and prevent ectopic expression of luciferase in the somatic tissues (Fig. 9). All four insulated mouse lines, however, expressed luciferase in testis despite the presence of the 50-bp insulator, indicating that the SP-10 insulator does not function as an enhancer blocker in testis.

Insulator Function in Vivo Is Not Orientation-dependent—Transient transfection experiments showed that the 50-bp insulator was totally ineffective as an enhancer blocker when placed in reverse orientation between the CMV enhancer and the reporter (Fig. 6). Biochemical analysis, however, showed that the SP-10 insulator functions by tethering the gene to the nuclear matrix. If this were the actual mode of insulator function, one would expect the orientation of the DNA sequence to be less important for function. In order to learn whether polarity is a requirement for enhancer-blocking function *in vivo*, we generated transgenic mice in which the SP-10 50-bp insulator was placed in the reverse orientation (−135/−186) between the CMV enhancer and the transgene (construct 4; Fig. 5*A*). The resultant mice showed somewhat leaky expression in somatic tissues, including brain, liver, kidney, and spleen (Fig. 10). The levels of luciferase expression in somatic tissues, however, were not as high compared with those in control mice lacking the insulator altogether (Fig. 8), suggesting only a partial compromise in insulator function. These results show that in the context of native chromatin, the 50-bp SP-10 insulator can function in either orientation. This is consistent with the observed mode of action of the SP-10 insulator (Fig. 1).

DISCUSSION

The present study is aimed at understanding how an insulator might regulate testis-specific transcription of the mouse *SP-10* gene. We show that an insulator located in the proximal promoter of the *SP-10* gene tethers the gene to the nuclear matrix in nongerm cells. This tethering sequesters the SP-10 core promoter and prevents the possibility of transcription in somatic tissues. In round spermatids, where the *SP-10*

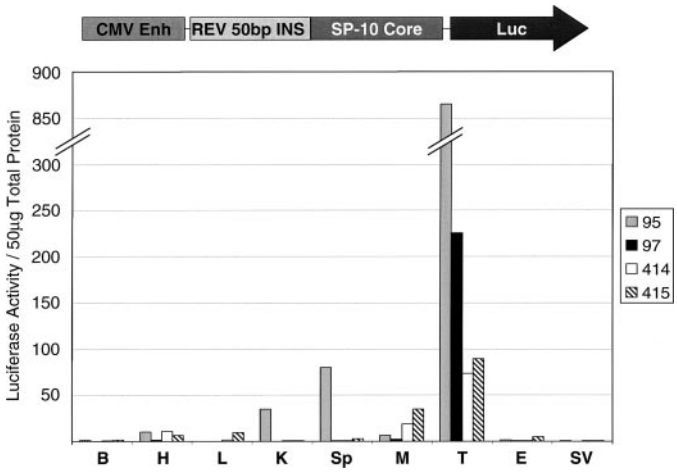


FIGURE 10. **Insulator function *in vivo* is not orientation-dependent.** Four transgenic mouse lines bearing the construct schematically shown above were analyzed. Reporter gene activity was observed in multiple tissues, but the levels were far less compared with uninsulated mice (Fig. 8), suggesting only a partial loss of enhancer-blocking activity. This is consistent with the mode of function of the SP-10 insulator by tethering to the nuclear matrix, which would be expected to occur in either orientation in a physiological context. Luciferase activities above background are plotted. *B*, brain; H, heart; *L*, liver; *K*, kidney; *Sp*, spleen; *M*, thigh muscle; *T*, testis; *E*, epididymis; *SV*, seminal vesicle.

gene is expressed, the gene is no longer tethered to the matrix. We suggest that differential tethering to the nuclear matrix mediated by the insulator regulates testis-specific transcription of the *SP-10* gene. Our experiments provide evidence for the involvement of TDP-43 in SP-10 insulator function. Finally, we show that a 50-bp subfragment of the SP-10 insulator functions as a minimal insulator in a physiological context. The SP-10 insulator lacks CpG dinucleotides and CTCF binding sites and offers the possibility to explore DNA methylation-independent modulation of insulator function in vertebrates.

Attachment to the nuclear matrix as a mechanism of insulator function has been proposed first by Gerasimova *et al.* (22), who showed that the *Drosophila gypsy* insulator is tethered to the nuclear periphery via interaction with insulator proteins suppressor of Hairy-wing (su(Hw)) and mod(mdg4). They proposed that insulators, by attaching the DNA to the nuclear periphery, separate unrelated enhancers and promoters into distinct chromatin loop domains in such a way that does not favor interaction between these regulatory elements. Subsequently, Yusufzai *et al.* (33) showed that vertebrate insulators also employ the same mechanism for enhancer blocking. They showed that the chicken β-globin insulator is tethered to a subnuclear structure by means of interaction with CTCF and its cofactor nucleophosmin and suggested that the mechanism of insulator function is conserved across invertebrates and vertebrates. Our present study using the mouse SP-10 insulator further confirms this idea (Fig. 1). Although insulators may vary in their nucleotide sequence and interacting proteins, attachment to the nuclear periphery or a subnuclear structure appears to be a conserved mechanism of insulator function. Given the complexity of action of enhancers, however, it is conceivable that there could be alternate modes of function of insulators.

The SP-10 insulator is facultative; therefore, its function must be modulated in the testis. What might be the nature of the modulation that untethers the SP-10 insulator from the nuclear matrix in the spermatogenic cells? Lack of CpG dinucleotides rules out DNA methylation as a possible mechanism. Instead, our results implicate TDP-43 in SP-10 insulator function. TDP-43 has been shown to be a transcriptional repressor and a splicing regulator (25, 26). Insulator function may be an additional role for TDP-43. It is not uncommon for transcription factors to have multiple roles in gene regulation. Importantly, the present study shows that TDP-43 is part of the nuclear matrix where a putative insulator protein would be expected to be present (Fig. 2). Our previous study showed that mutation of TDP-43 binding sites within the SP-10 insulator resulted in erratic expression (17). Consistent with this, knockdown of TDP-43 resulted in the release of SP-10-insulated transgene in a stable cell line model (Fig. 4). Taken together, this predicts that TDP-43, which is present in testis, must undergo either post-translational changes or splice variation in the male germ line to alter its function and permit *SP-10* gene expression. In fact, the present study shows that TDP-43 lacking the Gly domain can no longer be effective as an enhancer blocker (Fig. 3*B*). Our previous study showed that round spermatids express only the short form of TDP-43 mRNA compared with other cell types (Figs. 3 and 4 in Ref. 17). In addition, recent studies suggest that phosphorylation and ubiquitylation modify TDP-43 function in neurological cells (34). Our future studies will focus on the biology of TDP-43 in spermatogenic cells. It must be noted here that knockdown of TDP-43 in GC2 tissue culture cells did not result in expression of *SP-10* as determined by Western blots (data not shown). Our explanation for this is that in addition to release of repression, spermatid-specific activators will be required for the activation of the *SP-10* gene.

The significance of this study is that it characterized a 50-bp subfragment of the SP-10 insulator and showed its enhancer-blocking properties in a true physiological context. To our knowledge, this is the first demonstration in a mouse model that such a small 50-bp vertebrate insulator functions as a tissue-selective enhancer blocker. The evidence that the 50-bp minimal insulator can silence an otherwise ubiquitously expressed transgene in somatic tissues (Fig. 9) highlights the transcriptional regulatory strategies used by testis-specific genes. In light of its ability to act as an enhancer blocker in the heterologous context of the TK core promoter (Fig. 7), we propose a role for the 50-bp SP-10 insulator, which is conserved between mice and humans, in gene therapy vectors and for targeted gene delivery to testis, in particular.

The enhancer-blocking activity of the SP-10 insulator observed in the somatic tissues of transgenic mice (Fig. 9) begs the question as to whether the *SP-10* gene requires protection from the enhancer(s) of other gene(s) located nearby. In fact, on mouse chromosome 9 (NCBI, Map Viewer), the testis-specific *SP-10* gene is flanked by the ubiquitously expressed check point kinase 1 (*Chek1*) gene (35) on one side (less than 10 kb away) and a seminal vesicle-specific gene *Gm191* (symbol A630095E13Rik), which codes for SSLP-1 (secreted seminal vesicle Ly-6 protein 1) (36) on the other side (50 kb away). Thus, contiguous location of three genes with divergent tissue-spe-

cific expression patterns (1, 35, 36) warrants the presence of boundary elements capable of preventing inappropriate enhancer-promoter interactions. Therefore, it is conceivable that the SP-10 insulator characterized in the present study may play a role in protecting the *SP-10* gene from the influence of the enhancers of neighboring genes. The loci for mouse and human *SP-10* genes on chromosome 9 and chromosome 11, respectively, are syntenic, and the proximal promoters of mouse and human *SP-10* genes exhibit sequence homology (1), including the 50-bp insulator region. Evolutionary conservation of the sequence highlights the significance of SP-10 insulator function.

The SP-10 insulator, however, differs from other vertebrate insulators in terms of its location in the gene. Typically, insulators are located at gene boundaries upstream of the promoter and enhancer region. The HS4 chicken globin insulator is located several kilobase pairs upstream of the globin gene cluster and the LCR region (5). At the H19 locus, the imprinting control region containing the insulator is located far from the promoters of the imprinted *H19* and *Igf2* genes (10). Similarly, in the human *apoB* gene locus, the boundary element is located 55 kb upstream of the promoter (15). In contrast, the SP-10 insulator is located in close proximity to the core promoter (2). We hypothesize that the proximal promoters of some testis-specific genes have adapted insulator function during evolution. Testis-specific genes code for unique proteins that make up the acrosome, the neck, and the flagellum of spermatozoa and as such are not required by any other cell type. Sequestration of the core promoter by tethering to the nuclear matrix must have offered a fail-proof mechanism for these genes to remain silent in somatic tissues.

In conclusion, the present study provides a working model for testis-specific gene transcription. Although previous promoter analyses in transgenic mice have established that short proximal promoters of many testis-specific genes are sufficient for recapitulation of tight regulation shown by the endogenous genes (2, 37–41), the actual mechanism by which testis-specific promoters operate is poorly understood. Previous studies established a correlation between the methylation status of the promoter and gene expression for some testis-specific genes (42). A recent study implicated CTCF and Sp3 in mediating somatic repression of a testis-specific gene (43). Our study shows how an insulator located in a CpG-free proximal promoter can regulate testis-specific gene transcription. Specific *cis*-regulatory information on proximal promoters is expected to contribute toward restructuring gene regulatory networks (44–46). The compact nature of testis-specific promoters in general and the apparent ability of insulators to silence gene expression in somatic tissues (present study) prompt the prediction that insulators may be more prevalent in testis-specific gene promoters than has been realized.

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