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Wasted (*wst*) mice have 3-bp deletion in the PCNA promoter

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Abstract

Mice homozygous for the autosomal recessive wasted mutation (*wst/wst*) have abnormalities in T-lymphocytes and in the anterior motor neuron cells of the spinal cord, leading to sensitivity to ionizing radiation, hind limb paralysis, and immunodeficiency. This defect results in a failure to gain weight by 20 days and death at 28 days of age. Previous results from our group have shown that 1) wasted mice have little if any detectable PCNA protein or mRNA in thymus, but levels in liver, brain, and other tissues are similar to those in controls; and 2) the coding region for PCNA is the same in wasted mice and in control littermates. These observations gave rise to the present study, in which the PCNA promoter (620 nt 5' to the ATG codon) was sequenced for *wst/wst* mice, control littermates ($\bullet^{wst/+}$) and BCF₁ (or BALB/c $\times$ C57BL/6) F₁ controls. Sequence analysis revealed only one difference between *wst/wst* and BALB/c $\times$ C57BL/6 F₁ littermates: a 3-bp deletion in the 5' upstream region of the PCNA gene of wasted mice that was observed on only one allele or no alleles of normal littermates. The mutated sites in PCNA promoter from two litters plus two additional *wst/wst* and two known *wst/+* animals were screened with 8G and 11G probes, and each confirmed this pattern. The short term DNA segment encompassing the deletion (called the 11G element) was shown in gel shift experiments to bind a nuclear protein(s) present in a broad variety of cells including thymus and spleen nuclear extract from *wst/wst* and control mice. The mutated oligomer (called the 8G sequence) that was homozygous only in *wst/wst* mice was not able to bind the same nuclear protein(s). These experiments demonstrate that the wasted mutation (previously mapped to mouse chromosome 2) is caused by a 3-bp deletion in a T-cell-specific (and perhaps motor-neuron-specific) regulatory region of the PCNA gene also on mouse chromosome 2.

Wasted mice (*wst/wst*) were originally identified at the Jackson Laboratories as a mouse strain expressing a mutation in 25% of the offspring characterized by radiation sensitivity, neurologic dysfunction, and immune abnormalities.¹⁻¹³ Such mice were considered to be a model for the human disease ataxia telangiectasia (AT), but more recent studies have documented differences between the AT and the wasted syndrome.^{2,3,6} The wasted mouse phenotype has the following features: degeneration of anterior motor neurons in the spinal cord which leads to hind limb paralysis, diaphragm paralysis, and eventually death; T-cell immunodeficiency characterized by an absence of mature T-cells, an absence of T-dependent immune responses, and aberrant cytokine expression⁸⁻¹³; and sensitivity of T-cells to the killing effects of ionizing radiation and enhanced chromosomal abnormalities in bone marrow cells in response to γ -rays.¹⁻⁵ These features were all mapped to a single gene on chromosome 2.¹⁴

During the past several years, our group has attempted to clone the abnormal gene through several different approaches (differential screening of DNA libraries, differential display RT PCR) which proved unsuccessful. Abundant regulatory elements which confer cell type specificity and cell-cycle-specific expression have been identified in the promoter and intronic sequences of the gene.¹⁵⁻¹⁷ We recently reported drastically reduced expression of PCNA protein and mRNA in thymus tissues (but not brain or liver) of wasted mice relative to controls.¹⁵ PCR and sequencing analysis of the coding region revealed the presence of a PCNA pseudogene and a PCNA gene (on chromosome 2) that were not altered in wasted mice relative to controls. Because of the normal PCNA expression in wasted mouse liver and the absence of coding region abnormalities in the wasted gene, we designed experiments to analyze the promoter region of PCNA in wasted mice and their control littermates.

Figure 1 presents the sequence of the 535 to 570 region (according to Yamaguchi *et al.*, 1991)¹⁷ region of the PCNA promoter in wasted mice compared to the published C57BL/6 sequence of the gene.¹⁶⁻²³ A guanine segment (the 11G element) in normal mice is shortened by 3 bp only in affected *wst/wst* mice. Known *wst/+* heterozygotes (established by breeding experiments) carried one of each allele. The 3G bp was found in both alleles of *wst/wst* mice, in one allele of *wst/+* mice, and in no alleles of control BALB/c × C57BL/6 mice. Oligonucleotides specific for the 11G and 8G regions were synthesized and used to screen two litters from two pairs of *wst/+* parents. In all cases, *wst/wst* mice were discriminated from littermates by more intense hybridization to the 8G oligonucleotide. This will be useful as a screening tool for the mutation prior to onset of neurologic symptoms at 21 days of age.

The 11G element was noted in the literature to be within a larger region considered to be a negative regulatory region for T-cell expression.¹⁶ (It should be noted that gene regulation modules providing temporal and spatial gene expression specificity contain a mixture of both positive and negative regulatory elements.) We performed a sequence match analysis of the 11G sequence and surrounding nucleotides (AATGGGGGGGGGGGTAGGGG) by searching for other genes containing a similar region. Table 1 presents a partial listing of these genes, many of which have T-cell-specific functions, neural-specific functions, or are DNA-binding proteins. From this it appears that the 11G sequence is conserved among functionally related genes. Presence of the same 11G sequence in several homeodomain proteins is particularly interesting since homeobox proteins were indicated as important regulator of PCNA expression.¹⁷

Oligonucleotides containing the 11G sequence (AATGGGGGGGGGGGTAGGGG) and the wasted 8G sequence (AATGGGGGGGGGTAGGGGTGT) were synthesized and used in gel shift

assays to assess specific protein binding. These results, presented in Figure 2, reveal that the 11G element, but not the mutant 8G sequence, is capable of binding a protein(s) from nuclear extracts of normal as well as *wst/wst* mutant mice. Protein binding in spleen and thymus extract was observed (Fig. 3), as well as in extracts from HeLa and CHO cells (not shown). The 11G element binding is specific and can be reduced by competition with cold 11G oligonucleotide, but it is only slightly reduced by competition with 100-fold molar excess of the cold 8G element found in wasted mice. Nevertheless, wasted mice are not deficient in the binding protein(s), because extracts from wasted mice are also able to bind to the 11G element; it is the 8G mutated (wasted mouse) sequence that is unable to bind nuclear protein.

These data support the concept that the primary defect in wasted mice is a 3-bp deletion in a T-cell-specific (and possibly motor-neuron-specific) regulatory element in the 5' regulatory region of the PCNA gene. The gene is expressed normally in all tissues (including developing immature T-cells) and during development.¹³⁻¹⁵ As T-cells mature, another promoter element, the 11G element, is used. In wasted mice this element is defective, so that the PCNA gene can no longer be synthesized in mature T-cells; these cells die from an inability to enter the cell cycle. Since the 11G sequence strongly binds some protein(s) in all cell extracts that we tested so far, we propose that the 11G element is a positive regulatory element that acts in combination with T-cell-specific negative regulatory elements crucial for T-cell-specific PCNA gene expression; it can be "bypassed" in other cell types due to a redundancy of positive regulatory elements. The connection between altered PCNA expression and the defect in anterior motor neuron cells is not clear at this time, and experiments are underway to understand the role of PCNA in motor neuron cell survival. Since motor neurons are among the most radiation-sensitive cells in the nervous system,¹³ it is possible that these cells require PCNA for some as yet unidentified DNA repair or synthetic process. In a

tissue screening of mRNA for PCNA, brain was the only tissue with a low number of mitotic cells that showed very high levels of mRNA (more than liver).¹⁷ A recent report by Hernandez *et al.* (1997)²⁴ has documented abnormal PCNA expression in the spinal cord of Jimpy mutant mice.

It is noteworthy that PCNA has been shown to be associated with DNA repair and resistance to DNA damage. Shivji *et al.* (1992)²⁵ demonstrated that PCNA (known to be a subunit of DNA polymerase δ) is required for excision repair functions in mammalian cells. *Drosophila* mutants of the PCNA gene are sensitive to a variety of environmental mutagens.²⁶ It is not clear whether the radiation sensitivity of T-cells from wasted mice is related to cell cycle defects and/or due to the absence of repair activity. PCNA expression/mutation may provide a useful biomarker for radiosensitivity.

Sequence analysis of the human PCNA promoter has not shown evidence of an 11G sequence. However, several homopurine stretches have been found in the 5' portion and within several introns of the human PCNA gene. The affinity of the 11G-binding proteins for the homopurine stretch in the human PCNA gene is not known. Studies are underway to test this model.

Mammalian knock-out mutants for PCNA have generally been lethal both in cells in culture and in whole animals.^{29,30} The T-cells of wasted mice are not capable of being cultured *in vitro*⁵ and hence are difficult to study. The wasted mouse, however, with a developmental defect in the expression of PCNA, affords a unique opportunity for studies of the importance of PCNA in cellular functions and cellular interactions. A similar strategies for studying gene expression patterns, where control regions are modified in order to study the effects of spatial and temporal presence or absence of the gene product is already widely used in *D. melanogaster*, sea urchin, and other invertebrate organisms.^{27,28} The *wst* mice provide a rare and useful model for promoter modifications

that can be done on mammals.

Methods

Mice

Breeding pairs of mice that are heterozygous for the wasted (*wst*) gene were obtained from Jackson Laboratories and maintained along with C57BL/6 and BALB/c breeders in the animal facility in the Center for Mechanistic Biology and Biotechnology at Argonne National Laboratory. *wst/wst* mice were recognized by their failure to gain weight and neurologic symptoms evident at 21 days of age.¹⁻⁶

Sequencing analysis

DNA extractions and PCR amplification used established methods (15). Primers used for PCNA promoter PCR amplification and subsequent sequencing matched the published C57BL sequence.¹⁷ These sequences were

459 sense : 5' GGAGAAGCGTTTACGTTAAGAGG 3'

821 sense : 5' CGCAGCCCCGCCTTTGCATA 3'

877 antisense : 5' CCACCTCTCCTCAAGACCAAT 3'

996 antisense : 5' GGTGGCGGAGTTGTGGCGACTA 3'

Sequencing was carried out according to the manufacturer's instructions with the ThermoSequenase radiolabeled dideoxy terminators kit by Amersham.

Preparation of nuclear extracts

Cellular extracts were prepared by a modification of the methods described by Bodley *et al.*³¹ and Dignam *et al.*³² Several thymuses or spleens from wasted or BCF1 animals were homogenized

in ice-cold PBS at 0 °C. Cells were pelleted and washed with ice-cold PBS as needed and finally resuspended in 0.2 ml of ice-cold TEM buffer (10 mM TrisCl pH=8, 1 mM Na₂EDTA, 4 mM MgCl₂, 1 mM PMSF). Cells were incubated on ice for 15 min, then 0.2 ml of TEM buffer containing 2 M sucrose and 0.005% Triton X-100 was added. The mixture was pipetted several times and nuclei were precipitated for 0.5 min in an Eppendorf centrifuge. Nuclei were resuspended in one-half volume (relative to the packed cell volume) of ice-cold HS buffer (20 mM TrisCl, pH 8, 5 mM KCl, 1 mM MgCl₂, 20 mM NaHSO₃, 400 mM NaCl) and incubated on ice for 1 h. Nuclear debris was removed by centrifugation in an Eppendorf centrifuge for 5 min. The supernatant was dialyzed against buffer D (20 mM Hepes, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM Na₂EDTA, 1 mM PMSF, 0.5 mM DTT) and frozen at -70 °C.

Electromobility shift assays

20-mers containing eleven or eight Gs were end-labeled with T4 polynucleotide kinase. About 2×10^7 dpm (0.03 pmol of double-stranded oligonucleotide) were mixed with 3 μ l of nuclear extract for 15 min at room temperature in a buffer containing 4% glycerol, 1 mM MgCl₂, 0.5 mM Na₂EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM TrisCl, pH=7.5, and 0.08 mg/ml salmon sperm DNA. Specific and non-specific competitor double-stranded oligonucleotides (3 pmol) were mixed with the nuclear extracts and incubated for 10 min at room temperature before labeled oligonucleotides were added.

DNA/protein complexes were separated by 4% polyacrylamide gel electrophoresis. The running buffer was 0.5 x Tris-Borate. Gels were dried and exposed to PhosphorImager screens for 12 to 24 hours.

Sequences of oligonucleotides used for the assay were

11G sense strand : 5' AAATGGGGGGGGGGGTAGGGG 3'

8G sense strand : 5' AAATGGGGGGGGGTAGGGGTGT 3'

CHO nonspecific competitor (sense strand): 5' CTTGGTGGCTTCCAGTCT 3'

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Table 1. Genes with 10G/11G Element

Lymphocyte Related Genes

Mouse cyclophilin related protein (NK-cell-specific)	GGGGGGGGGGGGTAG
Human IGF1	GGGGGGGGGGGGTTT
Chicken myelomonocytic growth factor (CMGF)	GGGGGGGGGGGGTTG
Mouse interferon induced 15 kD protein	GGGGGGGGGGGGTAT
Mouse vasoactive intestinal peptide/cytokine response element	GGGGGGGGGGGGTTT
Human IL3 receptor α subunit	GGGGGGGGGGGGTAG
Human G protein coupled receptor kinase GRK4	GGGGGGGGGGGGTCG
Human CD40 ligand RNA	GGGGGGGGGGGGTTT
<i>Latimeria cholumnae</i> MHC class I	GGGGGGGGGGGGTTG
Mouse pp52/S37 (leukocyte/stromal cell specific)	GGGGGGGGGGGGTCT
Mouse Mort 1 (<i>fas</i> induced apoptosis associated protein)	GGGGGGGGGGGGCAG
Chicken lymphocyte homing receptor "link" protein	GGGGGGGGGGGGCTG
Mouse thymosin B4 (Ptmb 4)	GGGGGGGGGGGGCTC
Mouse class II cytokine receptor 4 (CRF20-4)	GGGGGGGGGGGGCAT
Mouse TNF receptor 2	GGGGGGGGGGGGTTG

Neural-Related Genes

Rat nerve growth factor receptor	GGGGGGGGGGGGTAG
Mouse NCAM neuronal adhesion gene	GGGGGGGGGGGGTTT
Chicken neuron glial adhesion molecule Ng CAM	*GGGGGGGGGGGATG
Mouse serotonin 2 receptor	GGGGGGGGGGGGTA*
Human BC200 neural specific RNA (cell body and dendritic location)	GGGGGGGGGGGGTTG
<i>Fugu rubripes</i> (pufferfish) neural adhesion neuropeptide	GGGGGGGGGGGGTCA

DNA Binding Proteins

Mouse PCNA	GGGGGGGGGGGGTAG
<i>Xenopus</i> transcription factor FB/b	GGGGGGGGGGGGTAG
Mouse msx 3 gene (homeobox gene)	GGGGGGGGGGGGTTT
Mouse c-myc	GGGGGGGGGGGGTCG
Chicken c-myc	GGGGGGGGGGGGTG*

Mouse zinc finger protein Zfx	GGGGGGGGGGGTTA
Mouse Hox 2.3	*GGGGGGGGGGTAC
Human Hox 2.1	*GGGGGGGGGGTTG
<i>D. melanogaster</i> antennapedia locus	GGGGGGGGGGGTTG
Mouse Sry locus	GGGGGGGGGGGTAG
Mouse p75 TNF receptor	GGGGGGGGGGGTTG
Mouse cyclin D	GGGGGGGGGGGTCC
Chicken helix loop helix transcription factor from the developing nervous system	GGGGGGGGGGGTCA
Mouse <i>msy-2</i>	GGGGGGGGGGGTAC
Mouse SOX1	GGGGGGGGGGGTGT
Mouse MRE binding transportation factor	GGGGGGGGGGGCAG
<i>D. melanogaster</i> Abd-B (bithorax complex)	GGGGGGGGGGGCAG
<i>C. elegans</i> HNF3 homeotic gene	GGGGGGGGGGGTTT

Other

<i>D. melanogaster</i> retinal degeneration gene C	GGGGGGGGGGGTTG
Human growth hormone	GGGGGGGGGGGTTG
Pig growth hormone 9D	GGGGGGGGGGGTTT
Mouse leptin	GGGGGGGGGGGTTC
<i>L. stagnalis</i> myomodulin	GGGGGGGGGGGTTG
Human Duchene muscular dystrophy (DMD)	GGGGGGGGGGGTTG
<i>Clupea haringus</i> troponin I (Tn I e)	GGGGGGGGGGGTCG
Pig myogenin	GGGGGGGGGGGTAG
Rat muscle specific enolase	GGGGGGGGGGGTCG
Chicken erythroid transport protein C1	GGGGGGGGGGGTCG
Chicken cytosolic adenylate kinase (AK1)	GGGGGGGGGGGTTG
Mouse anion exchange protein	GGGGGGGGGGGTAG
Rat endothelin 1	GGGGGGGGGGGTTG
<i>Canis familians</i> retinal outer membrane (rom 1) gene	GGGGGGGGGGGTCT
Chicken c- <i>erbA</i> thyroid hormone receptor	GGGGGGGGGGGCAG
Mouse myotonic dystrophy protein kinase DMR-mB15	GGGGGGGGGGGTA*

Figure Legend

- Figure 1. Sequence of 535 to 570 region of the mouse PCNA promoter compared with the same region in the wasted mouse (numbers are based on the published sequence of Yamaguchi *et al.*¹⁷ A * denotes a deleted nucleotide.
- Figure 2. Gel shift assays using nuclear extracts from (A) BCF₁ thymus, (B) *wst/wst* thymus, and (C) *wst/wst* spleen. These were bound to either a 8G or 11G element sequence (11G=AAATGGGGGGGGGGGGTAGGGG, 8G = AAATGGGGGGGGGTAGGGGTGT) and competed out with a 8G, 11G, or CHO (non-specific; CTTGGTGGCTTCCAGTCT) competitor.

C57BL/6 mouse 535 atatgaaatggggggggggtaggggtgtttaaata 570

wasted mouse 1 atatgaaatggggggggg***taggggtgtttaaata 35

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Figure 1.

BCF ₁ Thymus Extract							
Lane	1	2	3	4	5	6	7
Labeled oligo	G8	G8	G8	G11	G11	G11	G11
Cold competitor	0	G8	CHO	0	G8	G11	CHO

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Figure 2A.

<i>wst/wst</i> Thymus Extract						
Lane	1	2	3	4	5	6
Labeled oligo	G8	G8	G8	G11	G11	G11
Cold competitor	G8	G11	CHO	G11	G8	CHO

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Figure 2B.

<i>wst/wst</i> Spleen Extract						
Lane	1	2	3	4	5	6
Labeled oligo	G8	G8	G8	G11	G11	G11
Cold competitor	G8	G11	CHO	G11	G8	CHO

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Figure 2C.