ciation and GAP-like activity to changes in amounts of GTP-Ras may depend on the system under study and the physiological context in which it is examined.

It is likely that the lower proportion of Ras in the GTP-bound form in confluent cells is relevant to the phenomenon of contact inhibition. Ras is required for serum-induced growth of NIH 3T3 cells, and high activity of Ras, which results in cell transformation, can overcome contact inhibition. Our observations raise the possibility that the activity of Ras may help determine whether a cell will commit to continued proliferation. Normal concentrations of serum may be sufficient to induce mitogenic amounts of GTP-bound active Ras in subconfluent cells but not in confluent, contact-inhibited cells.

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Repression of the Insulin-Like Growth Factor II Gene by the Wilms Tumor Suppressor WT1

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The Wilms tumor suppressor gene *wt1* encodes a zinc finger DNA binding protein, WT1, that functions as a transcriptional repressor. The fetal mitogen insulin-like growth factor II (IGF-II) is overexpressed in Wilms tumors and may have autocrine effects in tumor progression. The major fetal IGF-II promoter was defined in transient transfection assays as a region spanning from nucleotides −295 to +135, relative to the transcription start site. WT1 bound to multiple sites in this region and functioned as a potent repressor of IGF-II transcription in vivo. Maximal repression was dependent on the presence of WT1 binding sites on each side of the transcriptional initiation site. These findings provide a molecular basis for overexpression of IGF-II in Wilms tumors and suggest that WT1 negatively regulates blastemal cell proliferation by limiting the production of a fetal growth factor in the developing vertebrate kidney.

Wilms tumor is a pediatric malignancy thought to arise when multipotent kidney blastemal cells fail to differentiate and instead continue to proliferate after birth (*1*). The occurrence of both sporadic and hereditary forms of Wilms tumor and the early age of bilateral kidney tumor onset suggest that Wilms tumors result when a predisposing germ line mutation is accompanied by a second mutation or loss of heterozygosity at the disease locus (*2*). Fine mapping of deletions in the chromosomal locus 11p13, which are associated with Wilms tumors, has culminated in the cloning of a potential tumor suppressor gene *wt1* (*3*). The *wt1* gene encodes a DNA binding protein with a serine- and proline-rich NH_2-terminus and four Zn^{2+} fingers (*3*, *4*). In the kidney, *wt1* mRNA is first detectable in the early stages of epithelial differentiation in the condensing mesenchymal cells or renal vesicle (*5*). The WT1 protein binds the DNA sequence GCGGGGGCG, a recognition element common to the early growth response (EGR) family of Zn^{2+} finger transcriptional activators (*4*, *6*). However, in contrast to the EGR transcription factors, WT1 behaves as a transcriptional repressor in transient transfection assays with synthetic promoter constructs (*7*). The biological significance of DNA binding and transcriptional regulation by WT1 is underscored by the observation that small deletions and point mutations in the WT1 Zn^{2+} fingers that abolish DNA binding have been detected in a number of Wilms tumors, especially in tumors associated with the Denys-Drash syndrome (*8*). The early expression of *wt1* during kidney development and WT1's capacity to function as a repressor of transcription suggest that the protein plays a key role in halting blastemal cell proliferation and in initiating a program of epithelial differentiation in the kidney.

Several lines of evidence suggest the involvement of the fetal mitogen insulin-like growth factor II (IGF-II) in the genesis of Wilms tumors. IGF-II is overexpressed in all Wilms tumors examined thus far (*9*). The Beckwith-Weidemann syndrome, a condition characterized cytogenetically by paternal duplications of the 11p15.5 chromosomal region, is associated with a predisposition for the development of Wilms tumors (*10*). This region contains the gene for IGF-II (*11*). Also, studies of Wilms tumors heterotypically transplanted in nude mice have shown that the tumor cells express the IGF type I receptor (*12*) and that antibodies that block this receptor inhibit tumor growth (*13*). These results suggest that IGF-II may function as an autocrine growth factor in Wilms tumor. Develop-

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mentally, IGF-II is abundantly expressed in kidney blastemal cells but not in differentiated epithelial cells (*14*), whereas the opposite pattern of expression is observed for *wt1* (*5*). The promoters that drive expression of the IGF-II fetal transcripts are GC rich (*15*) and contain several potential high-affinity binding sites for WT1, which raises the possibility that the IGF-II gene may be a target of WT1 transcriptional repression. We tested the hypothesis that WT1 interacts directly with the IGF-II promoter and represses this gene during nephrogenesis.

The reciprocal pattern of *wt1* and IGF-II expression during human kidney development was demonstrated by Northern (RNA) blot analysis of human fetal kidney RNA from different stages of development (Fig. 1, A and B). IGF-II expression was highest early in development and decreased dramatically, whereas WT1 expression increased with fetal development. Because of the heterogeneity of differentiating cells in the developing kidney, Northern analysis of total fetal kidney RNA represents a composite of the amount of total mRNA present and does not truly reflect the differences in gene expression at the cellular level. These results are consistent, however, with other in situ hybridization studies that show a mutually exclusive expression pattern for *wt1* and IGF-II (*14*).

The IGF-II gene is a complex transcription unit (*15*). Multiple transcripts are synthesized as a result of alternate promoter usage and the splicing of unique 5′ untranslated sequences to common coding exons (*16*). The 6.0-kb IGF-II transcript is the most abundant transcript in fetal kidney and Wilms tumor tissue and is the product of the P3 promoter. To test the hypothesis that the IGF-II gene is a target for WT1 repression, we first defined the region of the IGF-II P3 promoter necessary for high basal activity. Reporter constructs that contained promoter fragments were cloned 5′ of the bacterial chloramphenicol acetyltransferase (CAT) gene and transiently transfected into HepG2 cells (*17*). We chose HepG2 cells for these experiments because they express IGF-II at a high level that is comparable to the concentration expressed in fetal kidney (*9*, *15*) (Fig. 1C). The pattern of transcript abundance closely matched that of fetal kidney (Fig. 1, A and C), which suggests that promoter usage in these cells is similar. Available Wilms tumor cell lines (G401 and SK-NEP-1) do not express IGF-II (*18*) and are thus not representative of blastemal cells.

Reporter plasmids that included sequences up to nucleotide (nt) −295, relative to the transcription start site, showed high CAT activity, whereas deletions past nt −295 successively reduced promoter activity (Fig. 2A). We chose plasmid pP3[−295,+135], which spans from nt −295 to nt +135, for subsequent transient cotransfection assays with WT1 expression vectors (Fig. 2B). We inserted a human *wt1* cDNA 3′ of the cytomegalovirus (CMV) promoter-enhancer in the vector pCB6+ to create pCMVhWT (*7*). To make the control plasmid pCMVhWT-TTL, we inserted a synthetic DNA linker that consisted of stop codons in all three reading frames into the *wt1* expression vector at a unique Bam HI site that is 5′ to the Zn^{2+} fingers. When increasing amounts of WT1 expression vector were transfected with the IGF-II promoter reporter pP3[−295,+135], CAT activity decreased progressively to 10% of control amounts (Fig. 2, C and D). The control plasmid pCMVhWT-TTL had a minimal effect on CAT activity (Fig. 2, C and D).

The P3 promoter from nt −295 to nt +135 is 81% GC and contains multiple potential binding sites for WT1 (*15*, *19*). To examine whether the WT1 protein interacts directly with the IGF-II promoter, we performed electrophoretic mobility-shift assays with a synthetic WT1 Zn^{2+} finger protein (*4*). When the WT1 Zn^{2+} finger protein was incubated with an IGF-II P3 promoter fragment spanning from nt −295 to nt −98, two major complexes were formed (Fig. 3A), which suggests that two high-affinity WT1 binding sites are present 5′ of nt −98. An IGF-II promoter fragment covering nt −98 to nt +135 formed one dominant complex with the WT1 protein (Fig. 3A) at low protein concentration and a second, less prominent complex with larger amounts of protein. Thus, gel-shift analysis suggested that three high-affinity WT1 binding sites are present in the IGF-II P3 promoter. Consistent with these results, deoxyribonuclease (DNase) I footprinting demonstrated that multiple sequences in the IGF-II P3 promoter were protected from DNase I cleavage by the WT1 Zn^{2+} finger protein (Fig. 3B). The most complete protection was observed between nt −238 and nt −210 (site A), between nt −133 and nt

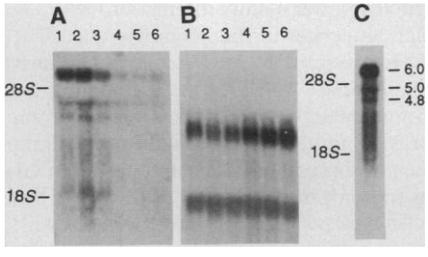

Fig. 1. Expression of IGF-II and *wt1* in human fetal kidney and HepG2 cells. Fetal kidney total RNA (**A** and **B**) or HepG2 cell total RNA (**C**) (10 μg per lane) were analyzed by Northern blot (*28*) with a probe that contained an IGF-II coding region (A and C) or with *wt1* cDNA (B). Kidney RNA samples (A and B) were from 10 (lane 1), 12 (lane 2), 14 (lane 3), 15 (lane 4), 17 (lane 5), and 19 (lane 6) weeks of gestation. The sizes of IGF-II transcripts (*16*) are shown in kilobases to the right of (C).

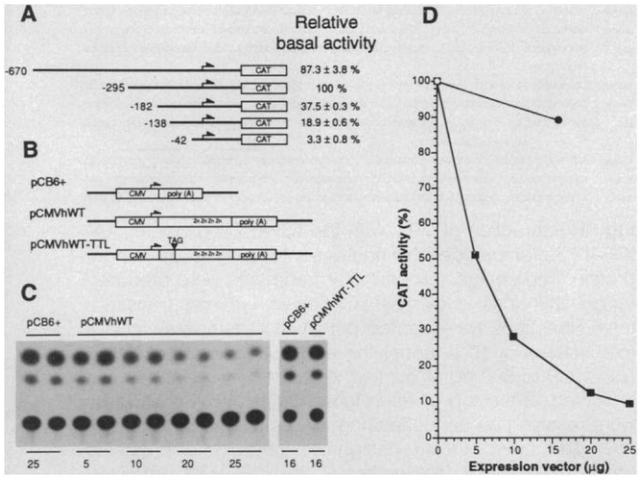

Fig. 2. Effect of WT1 on transcription from the IGF-II P3 proximal promoter region. (**A**) Definition of the IGF-II P3 promoter. Promoter constructs (*29*) were tested for basal activity in transient transfection assays in HepG2 cells (*17*). Basal CAT activity is expressed as a percentage of the strongest promoter construct pP3[−295,+135]. The mean ± SEM of three experiments is indicated for each construct. (**B**) Expression vectors used in cotransfection assays. We used the CMV-IE promoter in pCB6+ to drive expression of a *wt1* cDNA (pCMVhWT) or negative control pCMVhWT-TTL (*30*). (**C**) The reporter pP3[−295,+135] (1 μg) was cotransfected with 2 μg of internal control plasmid pON260 (*27*) and 0, 5, 10, 20, or 25 μg of pCMVhWT and a corresponding amount of pCB6+ to make up 30 μg of total transfected plasmid. The same amounts of pP3[−295,+135] and pON260 were also cotransfected with 16 μg of either pCB6+ or pCMVhWT-TTL. CAT assays were carried out as described (*17*). A representative thin-layer plate in an experiment performed with duplicate samples is shown. Numbers at the bottom of each lane show the amount in micrograms of the particular plasmid indicated at the top of each lane. (**D**) Plasmid pCMVhWT (■) repressed the IGF-II P3 promoter, whereas pCMVhWT-TTL (●) had little effect. Data points represent the average of two experiments performed with duplicate samples that gave similar results.

−119 (site B), and between nt +48 and nt +70 (site E) (Fig. 3C). These three WT1 sites contained the sequence GCGGGGGCG in either the forward (site B) or reverse (sites A and E) orientation (Fig. 3C). Comparison of the human (*15, 19*), mouse (*20*), and rat (*21*) IGF-II P3 promoters revealed a high degree of sequence similarity overall and perfect conservation of sites B and E (Fig. 3C), which suggests that these sites are functionally significant. Several other regions of the IGF-II promoter showed an intermediate amount of protection from DNase I cleavage (Fig. 3B), which suggests the presence of additional lower affinity sites for WT1.

To investigate the functional relevance of these sites, we cotransfected deletion derivatives of the IGF-II promoter with the pCMVhWT expression vector. With the exception of plasmids pP3[−42,+135] and pP3[−295,+35], WT1 repressed all constructs from 16.5 to 26% of their respective basal activity (Fig. 4A). Plasmid pP3[−295,+35], which lacks sequences from nt +35 to nt +135, maintained a high basal activity that was not repressed by WT1. Plasmid pP3[−42,+135] is essentially a minimal promoter with a TATA box, the transcription start site, and sequences downstream to +135. WT1 had little repressive effect on the low but reproducible promoter activity of this plasmid. These data suggested that binding of WT1 to sequences 5′ and 3′ of the IGF-II P3 transcription start site may be required to fully repress the IGF-II promoter. The importance of multiple WT1 binding sites positioned on each side of the start site was further substantiated by means of chimeric constructs of the herpes simplex virus thymidine kinase (HSV TK) promoter (*22*) and fragments of the IGF-II promoter. The starting plasmid pBLCAT2 contained the HSV TK promoter (nt −105 to nt +55) 5′ of the CAT gene and was not repressed in cotransfection assays with pCMVhWT (Fig. 4B). Insertion of an IGF-II promoter fragment spanning from nt −295 to nt −98 into pBLCAT2 5′ to the TK promoter (pTKAB) did not confer significant repression on pBLCAT2 nor did a 3′ insertion of an IGF-II promoter fragment spanning from nt +35 to nt +135 (pTKE). Insertion of IGF-II promoter fragments on both sides of the TK promoter (pTKABE), which reconstructed the relative positions of WT1 binding sites on either side of the IGF-II promoter, generated a reporter plasmid that was repressed in cotransfection assays with pCMVhWT (Fig. 4B). These results suggest that the effect of WT1 on IGF-II transcription is dependent on the presence of multiple binding sites positioned 5′ and 3′ relative to the site of transcriptional initiation.

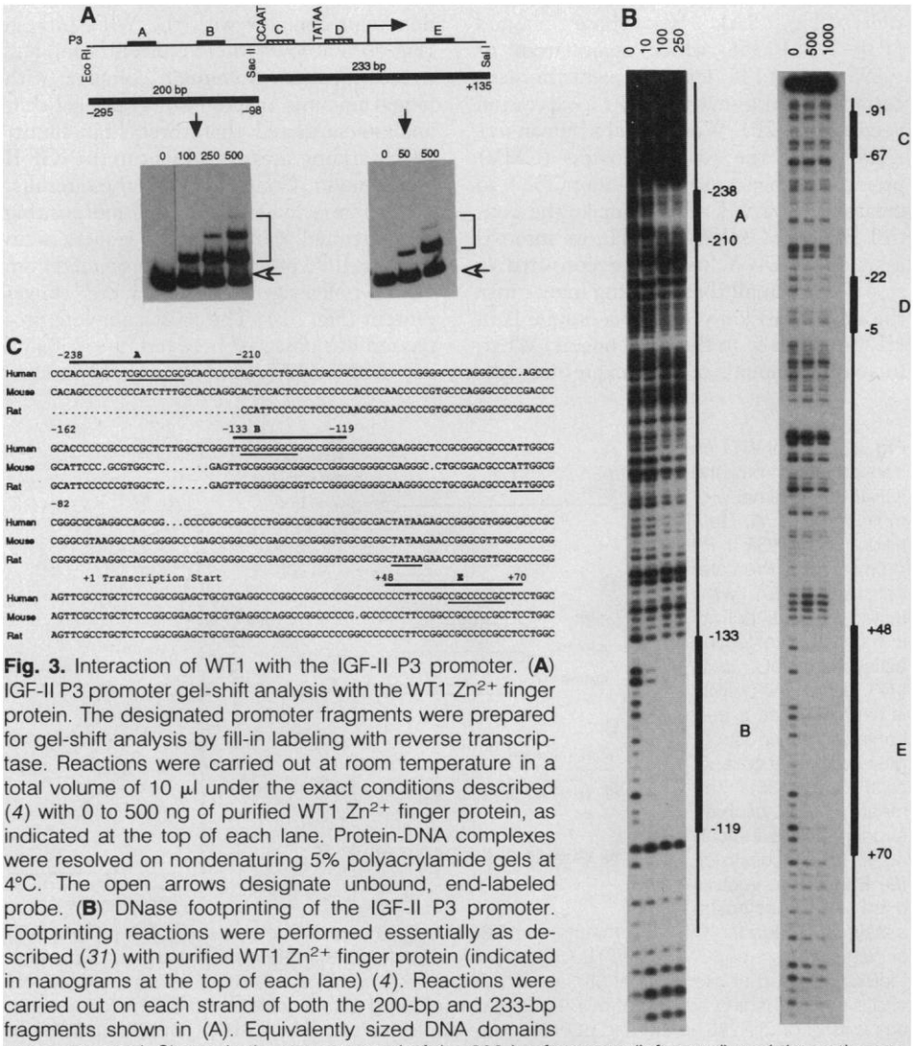

Fig. 3. Interaction of WT1 with the IGF-II P3 promoter. (**A**) IGF-II P3 promoter gel-shift analysis with the WT1 Zn^{2+} finger protein. The designated promoter fragments were prepared for gel-shift analysis by fill-in labeling with reverse transcriptase. Reactions were carried out at room temperature in a total volume of 10 μl under the exact conditions described (*4*) with 0 to 500 ng of purified WT1 Zn^{2+} finger protein, as indicated at the top of each lane. Protein-DNA complexes were resolved on nondenaturing 5% polyacrylamide gels at 4°C. The open arrows designate unbound, end-labeled probe. (**B**) DNase footprinting of the IGF-II P3 promoter. Footprinting reactions were performed essentially as described (*31*) with purified WT1 Zn^{2+} finger protein (indicated in nanograms at the top of each lane) (*4*). Reactions were carried out on each strand of both the 200-bp and 233-bp fragments shown in (A). Equivalently sized DNA domains were protected. Shown is the sense strand of the 200-bp fragment (left panel) and the antisense strand of the 233-bp fragment (right panel). (**C**) Conserved sequences in the IGF-II P3 promoter. Human (*15, 19*), mouse (*20*), and rat (*21*) promoters were aligned with the GCG sequence analysis program (*32*). Sequences protected from DNase I cleavage by WT1 are overlined and the WT1–EGR-1 consensus binding elements in the human sequence are underlined. Also underlined are a conserved TATA box at nt −31 and a reverse CCAAT box at nt −89.

We assessed the contribution of high- and low-affinity WT1 binding sites to the overall repression of the IGF-II promoter by introducing point mutations in the highest affinity WT1 binding sites (A, B, and E). Mutations that eliminate WT1 binding in site A (Fig. 4D) had little effect on the amount of repression by WT1 (Fig. 4C, pMA). This result is consistent with the WT1 repression of deletion construct pP3[−182,+135], which lacks site A (Fig. 4A). Mutations in site B (pMB), site E (pME), or sites A, B, and E together (pMABE) reduced the repression of the wild-type promoter from one-fifth of the basal activity to approximately one-half for these mutants (Fig. 4C). The differences in repression of these three mutants are not considered to be significant. The remaining repression by WT1 may be mediated through lower affinity interactions with GC-rich sequences in this promoter (Fig. 3B).

Repression of genes that encode positive regulators of cell growth is emerging as a common activity of a class of tumor suppressor proteins. Like WT1, the tumor suppressors p53 and Rb can function in vivo as transcriptional repressors (*23, 24*). Both wild-type p53 and Rb have been shown to repress the promoter for interleukin-6, an autocrine growth factor in a variety of malignancies (*25*). Other targets for Rb repression include growth factor–inducible genes such as c-*fos* and β-actin (*25*). On the basis of the data reported here, we propose that WT1 may function in a similar fashion to limit growth factor production. Functional loss of WT1 transcriptional repressor activity may result in continued synthesis of large amounts of IGF-II in

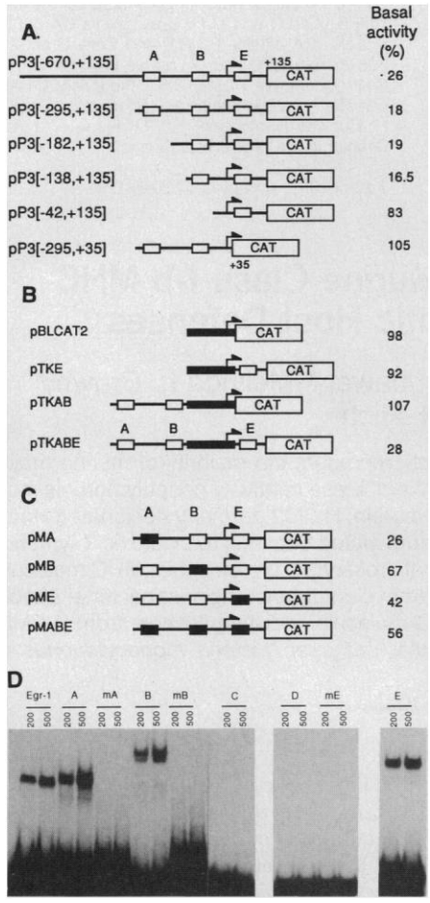

Fig. 4. The role of WT1 binding sites in the repression of the IGF-II P3 promoter. Transfections in (A), (B), and (C) were performed with 2 μg of reporter plasmid, 2 μg of internal control plasmid pON260, and 16 μg of either pCMVhWT or pCB6+. In (A), (B), and (C), repression is measured as the percentage of the respective basal CAT activity for each plasmid. Values represent the average of at least two experiments performed with duplicate samples that gave essentially identical results. (**A**) Repression of deletion constructs of the IGF-II P3 promoter (Fig. 2) by WT1. We made pP3[−295,+35] by digesting pP3[−295,+135] with Nae I and Sal I, blunt ending the strands with T4 polymerase, and then religating the plasmid. (**B**) Effect of WT1 on chimeric constructs of the HSV TK promoter and sequences from IGF-II P3. Reporter plasmids were generated by the insertion of an Eco RI–Sac II fragment (nt −295 to nt −98 of the P3 promoter) at the Bam HI site of pBLCAT2 (pTKAB), a Nae I–Sal I fragment (nt +35 to nt +135) at the Bgl II site (pTKE), or both insertions (pTKABE). (**C**) The effect of mutations in sites A, B, or E on the repression by WT1. We introduced mutations by PCR primer-directed mutagenesis (*33*) to alter site A from CGCCCCCGC to ATACCCCGC (pMA), site B from GCGGGGGCG to TATGGGGCG (pMB), and site E from CGCCCCCGC to TATCCCCGC (pME). Appropriate restriction fragments of the individual mutants were combined in the triple mutant (pMABE). (**D**) Mobility-shift assays of potential WT1 binding sites in the IGF-II promoter. Oligonucleotides corresponding to regions designated A, B, and E, as well as to other potential WT1 binding sites (C and D) of the IGF-II promoter, were assayed for their ability to interact with the WT1 Zn^{2+} finger protein in the amounts indicated at the top of each lane (in nanograms) (*4, 34*). Mutant oligonucleotides (mA, mB, and mE) and the consensus EGR-1 binding site have been described (*4, 34*).

kidney blastemal cells. The resulting unrestrained autocrine growth stimulation is a probable factor in the genesis of Wilms tumors.

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28. RNA samples were separated on a 1% formaldehyde-agarose gel, transferred to GeneScreen (DuPont) membranes, and probed with a random primer–labeled, 822-bp Pst I fragment of a human IGF-II cDNA [G. I. Bell *et al.*, *Nature* **310**, 775 (1984)] or a 1.8-kb Eco RI fragment of the WT33 (WT1) cDNA (*3*) in a solution containing 50% formamide, 7% SDS, 0.5 M sodium phosphate buffer (pH 7.2), 1 mM EDTA, 1% bovine serum albumin, and sonicated salmon sperm DNA (50 μg/ml) at 42°C. Blots were washed three times for 20 min at 65°C in 2% SDS, 1 mM EDTA, and 40 mM sodium phosphate buffer (pH 7.2) and then exposed overnight to Kodak X-OMAT film.
29. Restriction fragments of the P3 promoter from the λ phage clone λhIGF2-1 [G. I. Bell, D. S. Gerhard, N. M. Fong, R. Sanchez-Pescador, L. B. Rall, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6450 (1985)] were blunt-ended with T4 polymerase and cloned between blunt-ended Sac I and Hind III sites 5′ to a promoterless CAT gene in pGCAT-C [T. Frebourg and O. Brison, *Gene* **65**, 315 (1988)]. Restriction sites at nt −670 (Sac I), nt −295 (Eco RI), nt −182 (Sma I), nt −138 (Ava I), and nt −42 (Sac II), relative to the IGF-II P3 transcription start site, were used, as was a Sal I site as a common 3′ end at nt +135.
30. The WT1 expression vector pCMVhWT was constructed as described (*7*). Plasmid pCMVhWT-TTL was made from pCMVhWT by insertion of the synthetic oligonucleotide CTAGTCTAGACTAG (New England Biolabs), which contains a stop codon in each reading frame, at a unique Bam HI site (after amino acid 179 of the WT1 coding sequence).
31. M. R. Briggs, J. T. Kadonaga, S. P. Bell, R. Tjian, *Science* **234**, 47 (1986).
32. J. Devereaux, P. Haeberli, O. Smithies, *Nucleic Acids Res.* **12**, 387 (1984).
33. R. Higuchi, in *PCR Protocols: A Guide to Methods and Applications*, M. A. Innis, D. H. Gelfand, J. J. Sninsky, T. J. White, Eds. (Academic Press, San Diego, CA, 1990), pp. 177–183. Synthetic primer pairs with three-base mismatches (underlined) in the WT1-EGR1 consensus elements were as follows: CCACCCAGCCTTATCCCCGCGCA and GGGTGCGCGGGGATAAGGCTGGGT for site A, TCTTGGCTCGGGTTTATGGGGCG and CGCCCCATAAACCCGAGCCAAGA for site B, and TTCCGGCTATCCCCGCCTCCTGGC and GCCAGGAGGCGGGGATAGCCGGAA for site E. Primary polymerase chain reactions (PCRs) used a mutant primer and a 25-mer primer to either the SP6 or T7 promoter sequences in the vector flanking the insert. Successful reactions required Vent polymerase (New England Biolabs), 100°C melting cycles, and 80°C extension cycles. Equimolar amounts (0.1 μg) of primary products were combined for secondary PCRs with the vector primers alone. We replaced appropriate restriction fragments of the PCR products in pP3[−295,+135] to generate the mutant reporters and sequenced each plasmid to confirm the mutation and the absence of additional mutations.
34. Oligonucleotides used for the gel-shift analysis are listed below in the 5′ to 3′ direction with the G-rich strand shown. Underlined nucleotides represent changes present in the mutant oligonucleotides as designated: A, (−215) GGGTGCGCGGGGGCGAGGCTGGGT (−238); mA, GCG → ATA; B, (−136) GGTTGCGGGGCGGGGCCG (−119); mB, GCG → TAT; C, (−91) CCATTGGCGCGGGCGCGAGGCCAGC (−67); D, (−25) AGCCGGGCGTGGGCGCCCGCAGTT (−2); E, (+71) CAGGAGGCGGGGGCGGCCGGAAGG (+48); and mE, GCG → ATA. Complementary oligonucleotides were 100% homologous

to the sequences shown. Preparation of end-labeled double-stranded probes was performed as described (*4*).

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Specialized Role for a Murine Class I-b MHC Molecule in Prokaryotic Host Defenses

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Although nonclassical (class I-b) gene products represent the majority of murine major histocompatibility complex (MHC) genes, the role of these relatively nonpolymorphic molecules remains uncertain. Recently, one such protein, H-2M3 (formerly designated Hmt), was shown to bind and specifically present *N*-formylated peptides to cytotoxic T lymphocytes. Because *N*-formylation is characteristic of prokaryotic proteins, this MHC molecule may be especially adapted for a role in the mammalian defense against bacterial attack. The current studies demonstrate that an MHC molecule, indistinguishable from H-2M3, presents antigens derived from the intracellular pathogen *Listeria monocytogenes* to *Listeria*-specific CD8$^+$ cells.

Murine listeriosis has provided a model for studying the role of CD8$^+$ T cells in the host defense against intracellular pathogens (*1*). Although animals infected with *L. monocytogenes* generate a humoral response to infection, antibodies cannot damage invading bacteria because of their intracellular location. By contrast, peptide fragments derived from intracellular *L. monocytogenes* can be processed and presented in association with class I MHC products (*2, 3*), facilitating the lysis of infected cells by *Listeria*-specific CD8$^+$ cytotoxic T lymphocytes (CTLs). Because infected cells represent a major site for bacterial proliferation, this selective destruction can be an effective strategy by which the host can contain *L. monocytogenes* (and other intracellular pathogens) in vivo (*1, 4*).

The presentation of some prokaryotic antigens to mouse CD8$^+$ T cells is restricted by classical class I MHC products (H-2K, D, or L), implying that these molecules contribute to peptide recognition (*5*). However, a subpopulation of *Listeria*-specific CD8$^+$ T cells selectively lyses *Listeria*-treated allogeneic target cells lacking any shared K, D, or L allele (*6, 7*). Nonetheless, β_2-microglobulin is required for *Listeria* antigen recognition by these cells, implying that a class I MHC product is required in antigen presentation (*3*). This pattern suggests that these effectors recognize *Listeria*-derived peptides associated with a monomorphic, nonclassical class I-b MHC product (*8*) distinct from H-2K, D, or L.

The murine class I-b product H-2M3^a (*9*) is a particularly attractive candidate for *Listeria*-antigen presentation (*10*). This molecule, which is expressed by all common strains of laboratory mice, binds *N*-formyl peptides derived from the mitochondrially encoded reduced form of nicotinamide adenine dinucleotide (NADH) dehydrogenase subunit 1 (ND1) (*11*), forming the minor histocompatibility antigen Mta (maternally transmitted antigen). Using a competitive binding assay, we have demonstrated that this molecule preferentially binds peptides bearing an *N*-formyl group (*12*). By contrast, acetylated and unmodified peptides of identical sequence bind poorly or not at all to H-2M3^a. Because *L. monocytogenes* (and other prokaryotes) initiate protein synthesis with *N*-formylmethionine (*13*), we and others have speculated that H-2M3 may be adapted for the task of presenting microbial peptides to CD8$^+$ effectors (*11*). The current studies establish that a class I MHC product indistinguishable from H-2M3^a is responsible for the apparent "MHC-unrestricted" presentation *Listeria* antigens to CD8$^+$ T cells.

Listeria-specific CTLs derived from C57BL/6 (B6) mice (H-2K^b, D^b, H-2M3^a) have been characterized as MHC-unrestricted because they can lyse *Listeria*-treated target cells obtained both from syngeneic mice and from a range of allogeneic strains (*6, 7*). However, target cells from all strains previously tested express H-2M3^a (*10*). To examine whether *Listeria*-immune CD8 effectors are restricted by H-2M3^a, we measured the capacity of these effectors to lyse antigen-treated macrophages from B10.CAS2 mice. This mouse strain (produced by backcrossing a *Mus musculus castaneus* H-2 complex into a B10.BR, Mta$^+$ background) expresses H-2M3^b, a rare nonfunctional H-2M3 allele (*14*). Although the CTLs used in these experiments specifically lysed *Listeria*-treated macrophages from allogeneic BALB/c mice (Table 1), they could not destroy comparable targets prepared with B10.CAS2 macrophages (Table 1). The apparent absence of lysis of B10.CAS2 targets did not reflect intrinsic resistance to lysis by CTLs, because the same targets were readily lysed in the presence of an antibody to CD3, which induces antigen-independent, CTL-mediated lysis (*15*).

Table 1. Lysis of B6, BALB/c, and B10.CAS2 macrophages (M0) by *Listeria*-immune CD8 cells from B6 mice. Thioglycollate-induced peritoneal macrophages in experiment 1 from B6 (H-2K^b, D^b, H-2M3^a) and BALB/c (H-2K^d, D^d, H-2M3^a) or in experiment 2 from B6 and B10.CAS2 (H-2K^{cas2}, D^{cas2}, H-2M3^b) mice were plated at 3 × 10^4 cells per well in 96-well plates and allowed to adhere overnight at 37°C. Monolayers were then incubated with medium alone, with a partially purified *Listeria* membrane preparation (6 μg/ml) (*3*), or with an antibody to CD3 (2C11) (3 μg/ml) for 18 hours. At that time, monolayers were washed, treated with brefeldin (1 μg/ml) to block further antigen processing, and incubated with *Listeria*-immune CD8 cells (*3*), 9 × 10^4 cells per well, for an additional 6 hours at 37°C. *Listeria*-specific lysis of macrophage targets was quantitated with the use of a neutral red uptake technique (*3, 18*). The triplicate sets of numbers in experiment 2 represent the results of three separate, paired experiments.

M0s incubated with	Lymphocyte-mediated lysis (%) of M0 targets from		
	B6	BALB/c	B10.CAS2
	Experiment 1		
Medium	0	0	—
Listeria membrane antigen	43	50	—
	Experiment 2		
Medium	7, 11, 0	—	0, 0, 0
Listeria membrane antigen	54, 22, 52	—	0, 0, 0
Anti-CD3 antibody	61, 52, 68	—	69, 55, 47

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