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Functional Analysis and Nucleotide Sequence of the Promoter Region of the Murine *hck* Gene

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The structure and function of the promoter region and exon 1 of the murine *hck* gene have been characterized in detail. RNase protection analysis has established that *hck* transcripts initiate from heterogeneous start sites located within the *hck* gene. Fusion gene constructs containing *hck* 5′-flanking sequences and the bacterial Neo^r gene have been introduced into the hematopoietic cell lines FDC-P1 and WEHI-265 by using a self-inactivating retroviral vector. The transcriptional start sites of the fusion gene are essentially identical to those of the endogenous *hck* gene. Analysis of infected WEHI-265 cell lines treated with bacterial lipopolysaccharide (LPS) reveals a 3- to 5-fold elevation in the levels of endogenous *hck* mRNA and a 1.4- to 2.6-fold increase in the level of Neo^r fusion gene transcripts, indicating that *hck* 5′-flanking sequences are capable of conferring LPS responsiveness on the Neo^r gene. The 5′-flanking region of the *hck* gene contains sequences similar to an element which is thought to be involved in the LPS responsiveness of the class II major histocompatibility gene $A_\alpha k$. A subset of these sequences are also found in the 5′-flanking regions of other LPS-responsive genes. Moreover, this motif is related to the consensus binding sequence of NF-κB, a transcription factor which is known to be regulated by LPS.

Murine *hck* (19, 42, 60) is a member of a growing family of cytoplasmic protein tyrosine kinases which include *src*, *lck*, *yes*, *lyn*, *fgr*, *fyn*, and *blk* (8, 10, 24, 25, 30, 31, 36, 55, 58). *src*-related genes encode proteins with a highly conserved C-terminal catalytic domain, a separate region with structural similarity to phosphatidylinositol-dependent phospholipase C (31, 50) and p21^{ras} GTPase-activating protein (53, 54), and a unique N-terminal domain, which includes an N-terminal myristylated glycine.

There is a strong association between tyrosine kinase activity and transforming activity of several *src*-related proteins. The ability of *src* family members, under certain conditions, to transform cells in vitro suggests that these enzymes play a role in the signal transduction pathways which regulate cell growth (20). The kinase activity of pp60$^{c\text{-}src}$ and other *src*-related tyrosine kinases is regulated by phosphorylation at two conserved tyrosine residues (Tyr-416 and Tyr-527 in pp60$^{c\text{-}src}$). For example, dephosphorylation of Tyr-527 in pp60$^{c\text{-}src}$ leads to a substantial elevation in protein kinase activity (4–7). Indeed, replacement of this tyrosine with phenylalanine results in both elevated pp60$^{c\text{-}src}$ kinase activity and increased transforming ability (3, 26, 41, 45). The analogous mutants of p56^{lck} and p59^{hck} also have enhanced transforming activity in fibroblasts (1, 29, 59). Although this correlates with an elevation in apparent kinase activity for p56^{lck} (29), surprisingly, no detectable change in p59^{hck} kinase activity accompanies cellular transformation (59).

Regulated expression of members of the *src* family of protein tyrosine kinases also occurs at the level of transcription. For *hck*, transcripts have only been detected in hematopoietic tissues and cell lines of the myeloid and B lymphoid lineages (19, 42, 60). Moreover, an increase in the levels of steady-state *hck* mRNA has been observed following the induced differentiation of the human granulocytic and monocytic cell lines ML-1, HL-60, and U937 (42) and the murine myelomonocytic cell line WEHI-3B (19). Induction of *hck* mRNA has also been noted in normal human macrophages (61) and murine bone marrow macrophages (our unpublished data) following exposure of cells to bacterial lipopolysaccharide (LPS). These observations suggest a role for *hck* in some aspect of differentiation and/or functional activation of these cells.

To investigate the transcriptional regulation of the murine *hck* gene, we have isolated its putative promoter region and determined the sites of transcriptional initiation. We have also shown that a genomic fragment which includes 646 base pairs (bp) of 5′-flanking DNA is functionally active in cultured hematopoietic cells and capable of conferring LPS responsiveness on a reporter gene.

MATERIALS AND METHODS

Genomic library screening and DNA sequencing. A murine BALB/c genomic library (22) was screened with a radiolabeled *hck*-specific probe corresponding to the 5′-most 319 nucleotides of the *hck* cDNA (19). Positively reacting clones were purified to homogeneity and bacteriophage DNA extracted (27) and subcloned into M13mp18 and M13mp19 vectors (34). Subclones were sequenced by the method of Sanger et al. (46).

Cell lines. The cell lines WEHI-265 (56), RAW8 (44), FDC-P1 (9), and PU5-1.8 (43) were maintained in Dulbecco modified Eagle medium in the presence of 10% fetal calf serum. This medium was supplemented with WEHI-3-conditioned medium (a source of interleukin-3) for FDC-P1, or 10^{-4} M asparagine for PU5-1.8. The NIH 3T3 (21) and ψ2 (28) cell lines were grown in Dulbecco modified Eagle medium supplemented with 10% newborn calf serum.

RNase protection mapping. The conditions used to determine the transcriptional start sites of the *hck* gene were essentially identical to those described by Melton et al. (33).

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Briefly, an mRNA complementary and uniformly labeled riboprobe designated SB was generated by in vitro transcription of the *Spe*I-*Bam*HI genomic *hck* fragment shown schematically in Fig. 2A. The probe (2×10^5 to 5×10^5 cpm) was hybridized for 16 h at 45°C to poly(A)$^+$ RNA (1 to 2 μg) from each cell line. After digestion of the samples with RNase A (40 μg/ml) and RNase T_1 (2 μg/ml) at 30°C, reactions were terminated with proteinase K (150 μg/ml) and sodium dodecyl sulfate (1%), and the products were extracted successively with phenol, phenol-chloroform, and chloroform. Samples were precipitated with ethanol in the presence of 10 μg of *Escherichia coli* tRNA and washed with 70% ethanol before being dissolved in 2 μl of TE. Samples were fractionated on denaturing 6% acrylamide gels by using an M13 sequencing ladder as a molecular size standard.

A second riboprobe, designated SBB, for distinguishing endogenous *hck* transcripts from viral fusion transcripts, was generated by in vitro transcription of the composite genomic-cDNA fragment shown in Fig. 4A. The riboprobe template contains the *Spe*I-*Bam*HI *hck* genomic DNA fragment described above and a contiguous 132-bp *Bam*HI *hck* cDNA fragment (19). As a quantitative measure of the amount and integrity of poly(A)$^+$ RNA in the LPS induction experiment (see Fig. 5), samples were simultaneously analyzed with a β_2-microglobulin probe. The probe was generated by in vitro transcription of a 37-bp murine *Eco*RI-*Pst*I β_2-microglobulin cDNA fragment (40). The conditions used for the synthesis, hybridization, and RNase digestion of both these probes were identical to those described above.

Construction of a recombinant *hck*-Neo^r retrovirus. The putative *hck* promoter, including 0.65 kilobase pairs (kb) of 5′-flanking sequences, was introduced into the *Bgl*II site immediately preceding the bacterial Neo^r gene (49) in the retroviral vector RV431 (E. Stanley and A. R. Dunn, unpublished data). This vector (see Fig. 3A) has a deletion in its 3′ long terminal repeat (LTR) which removes the enhancer and promoter elements. Following a single cycle of replication, this deletion is transferred to the 5′ LTR and the Neo^r gene is placed under the control of the putative promoter.

DNA transfection and viral infection of hematopoietic cell lines. A retroviral vector plasmid, containing the putative *hck* promoter and 646 bp of 5′-flanking DNA, was transfected into ψ2 cells (28) by the calcium phosphate procedure of Graham and Van der Eb (17) as modified by Parker and Stark (39). Stable ψ2 producer lines were selected in the presence of 400 μg of the neomycin analog G418 (geneticin; Sigma Chemical Co.) per ml. The viral titers of several ψ2-packaging cell lines were determined by dot blot hybridization (S. Gerondakis, personal communication; data not shown). A cell line, designated XB4, was judged to have the highest viral titer and was used for all subsequent experiments.

To infect target cells, virus producer cell lines were cocultivated at a density of 2×10^5 cells per 5-cm dish with an equivalent number of either FDC-P1 or WEHI-265 cells until the producer cells were confluent (between 48 and 72 h). The nonadherent FDC-P1 and WEHI-265 cells were plated in 0.3% agar containing the appropriate growth medium and selected in G418 (1 mg/ml) for approximately 14 days. Colonies of G418-resistant FDC-P1 cells (designated F1 through F15) and WEHI-265 cells (XW1-4), were isolated and expanded in liquid culture before poly(A)$^+$ RNA was isolated. To achieve infection, it was necessary to treat WEHI-265 cells with 1 μg of tunicamycin (Sigma) per ml for 24 h prior to cocultivation.

Preparation of RNA and Northern hybridization analysis. Poly(A)$^+$ RNA was prepared by the procedure of Gonda et al. (16). Northern (RNA) hybridization analysis was conducted as previously described (27) by using an mRNA complementary probe generated by in vitro transcription of a pGEM plasmid (Promega Biotec) containing the complete Neo^{r}gene (49). Filters were washed at 68 to 75°C in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (27) in the presence of 0.3% sodium dodecyl sulfate and subjected to autoradiography.

Stimulation of WEHI-265 cells with LPS. To determine whether the *hck* promoter region contained sequences responsive to bacterial LPS, G418-resistant WEHI-265 clones XW1, XW2, and XW4 were incubated in the absence or presence of 1 μg of LPS (Difco Laboratories) per ml for 8 h. Poly(A)$^+$ RNA was extracted from cells, and an equal amount (2 μg) from treated and untreated cells was subjected to RNase protection analysis with *hck* (see Fig. 4A) and β_2-microglobulin (40) riboprobes (see above). The cell culture medium was determined to be essentially free of endogenous LPS (less than 0.1 ng/ml) by the *Limulus* amebocyte lysate assay (Commonwealth Serum Laboratories, Parkville, Australia), kindly performed by G. Vairo, University of Melbourne.

To quantitate the relative amounts of endogenous *hck* transcripts and Neo^r fusion gene transcripts, radiolabeled protected fragments were excised from the dried acrylamide gel and subjected to liquid scintillation counting. The values obtained were standardized with respect to β_2-microglobulin-specific hybridization.

Nucleotide sequence accession number. The GenBank accession number for the nucleotide sequence of the promoter region of the murine *hck* gene is M34946.

RESULTS

Structure of the 5′ end of the murine *hck* gene. To isolate the *hck* promoter and 5′-flanking sequences, we screened a murine genomic library with a radiolabeled *Eco*RI-*Sph*I fragment corresponding to the first 319 bp of the murine *hck* cDNA (19). One positive clone, designated 9B1, contained approximately 12 kb of chromosomal DNA. We determined the nucleotide sequence of a 1.1-kb *Xho*I-*Kpn*I genomic subclone of 9B1 (Fig. 1), which hybridized to sequences found within the 5′ terminus of the *hck* cDNA. This sequence contained a 167-bp region which was identical to 5′ untranslated sequences of the *hck* cDNA (19) and included a consensus splice donor site (35), defining the 3′ boundary of exon 1. On the basis of restriction map data and Southern blot analysis of clone 9B1 with oligonucleotide probes (data not shown), it was concluded that an intron of at least 8 kb resides between the untranslated exon shown in Fig. 1 and the exon containing the translational start codon.

Identification of the transcriptional start site of the *hck* gene. Previous studies have shown that *hck* is expressed predominantly in cells of the B-lymphoid and myeloid-macrophage lineages (19, 42, 60). To determine the transcriptional initiation sites of the *hck* gene, we performed RNase protection with poly(A)$^+$ RNA from two macrophagelike cell lines (WEHI-265 and PU5-1.8) and a B-lymphoma cell line (RAW8), which are known to express *hck* transcripts. The results of this analysis, using the mRNA complementary probe SB, are shown in Fig. 2. The radiolabeled probe was transcribed from a plasmid containing a genomic *hck* fragment (*Spe*I-*Bam*HI), which includes untranslated sequences from exon 1 and the presumptive 5′-flanking region. Figure 2A is a schematic representation of the RNA probe, the

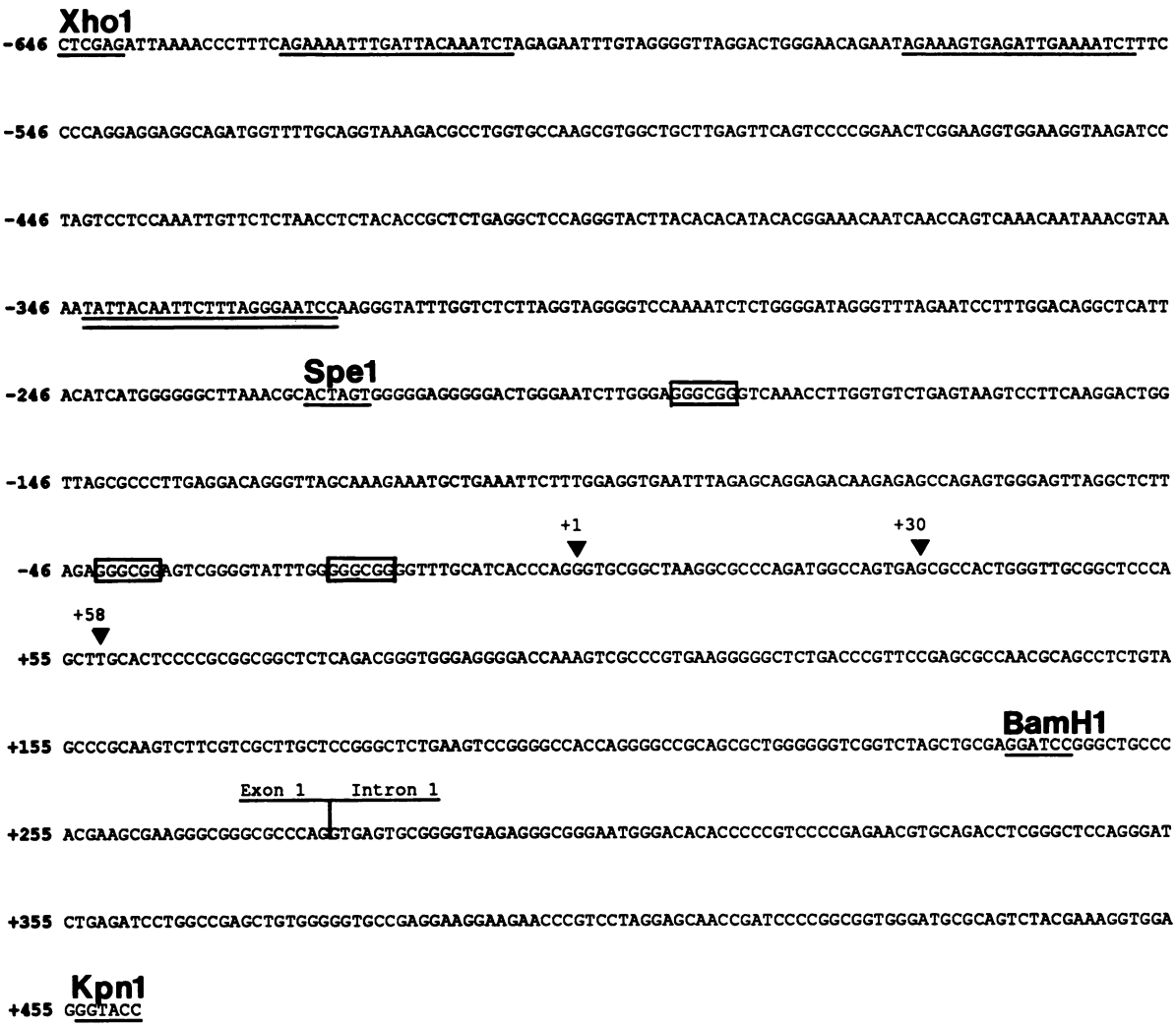

FIG. 1. Nucleotide sequence of the promoter region of the murine *hck* gene. Both strands of the 1.1-kb *Xho*I-*Kpn*I fragment shown were sequenced by the chain termination method of Sanger et al. (46). Key restriction sites and the junction between exon 1 and intron 1 are indicated. Major transcriptional initiation sites, at positions +1, +30, and +58, are indicated (▼); the 5′-most of these is defined as +1. The positions of three potential Sp1-binding sites at positions −191, −43, and −22 are boxed. An imperfect 21-bp repeat is underlined. A 23-bp element at position −344, which bears similarity to sequences found in the 5′-flanking regions of genes encoding various immunoregulatory proteins, is doubly underlined (also see Fig. 6).

putative *hck* mRNA to which it would hybridize, and the products expected to resist RNase digestion.

The results of the RNase protection analysis are presented in Fig. 2B. Transcripts from the cell line AFD5 (lane 1), which stably expresses murine *hck* cDNA (19) from a retroviral long terminal repeat (E. S. and A.R.D., unpublished data), protect a fragment of 135 nucleotides (nt). This fragment is the size expected for the 5′ terminus of the *hck* cDNA (19), indicating the precision of the RNase protection assay. Transcripts from the cell lines PU5-1.8 (lane 2), RAW8 (lane 3), and WEHI-265 (lane 4) protect a cluster of discrete fragments ranging in size from 188 to 245 nt. These fragments define several transcriptional start sites including those at positions +1 (the 5′ site), +30, and +58 (Fig. 1). Poly(A)$^+$ RNA from FDC-P1 (lane 6) and NIH 3T3 (lane 7) cells was not protected, confirming previous data that these cells do not express *hck* mRNA (19; our unpublished data).

To determine whether other transcriptional start sites or splice sites were presented in the 423-bp region between the *Xho*I and *Spe*I restriction sites (Fig. 1), samples of PU5-1.8, RAW8, and WEHI-265 RNA were analyzed with a radiolabeled RNA probe complementary to the entire *Xho*I-*Bam*HI region. The fragments protected by this probe (data not shown) were identical to those observed in Fig. 2B, indicating the absence of other transcriptional start sites or splice sites within this region.

The major *hck* mRNA start sites (determined above) are depicted in Fig. 1 in the context of the 5′-flanking DNA of the *hck* gene. Like many genes in which transcription is initiated from heterogeneous sites, the *hck* promoter region is G+C rich and includes GC boxes which conform to the consensus sequence for Sp1-binding sites (11, 12, 23, 32). Three such GC boxes (indicated by rectangles in Fig. 1) are present in the 5′ end of the murine *hck* gene at positions −191, −43, and −22. An imperfect 21-bp repeat is located at the 5′ end of the sequence shown (Fig. 1). The significance of this element to the regulation of transcription of the *hck* gene remains to be established. A 23-bp element (doubly underlined in Fig. 1) located between positions −344 and −322 has extensive homology with elements present in the 5′-flanking

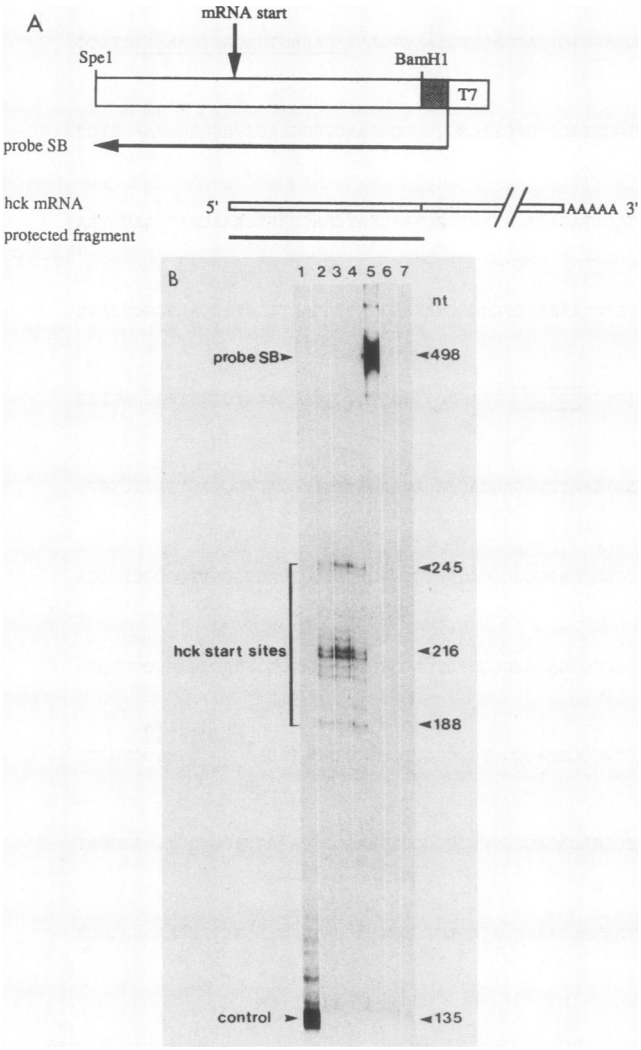

FIG. 2. Determination of *hck* transcriptional start sites by RNase protection analysis. (A) A schematic representation of the riboprobe SB and its relationship to the *hck* mRNA start site(s). A plasmid containing an *SpeI-BamHI* genomic *hck* fragment (Fig. 1) was transcribed with T7 polymerase to generate the antisense probe designated SB. The probe contains sequences known to be present in the *hck* 5′ untranslated region and putative 5′-flanking region as well as some plasmid sequences (▨). The position of the potential mRNA start site(s) is indicated. Fragments of probe which would be protected by *hck* mRNA are also indicated. (B) Electrophoretic analysis of fragments protected from RNase digestion by poly(A)$^+$ RNA (2 μg) from the cell lines AFD5 (lane 1), PU5-1.8 (lane 2), RAW8 (lane 3), WEHI-265 (lane 4), FDC-P1 (lane 6), and NIH 3T3 (lane 7). The molecular sizes of the undigested probe (lane 5) and the major protected species are given in nucleotides. A fragment protected by RNA from AFD5 cells (control) is also indicated. The molecular sizes were estimated by comparing the mobilities of protected fragments with those of bands in an M13 sequence ladder (not shown).

region of a number of genes encoding immunoregulatory factors which are transcriptionally regulated by LPS (see Fig. 6 and Discussion).

Analysis of transcriptional start sites in stably infected hematopoietic cells. The retroviral vector RV431, shown schematically in Fig. 3A, was designed to test for functional activity of the *hck* promoter. The vector comprises *hck*

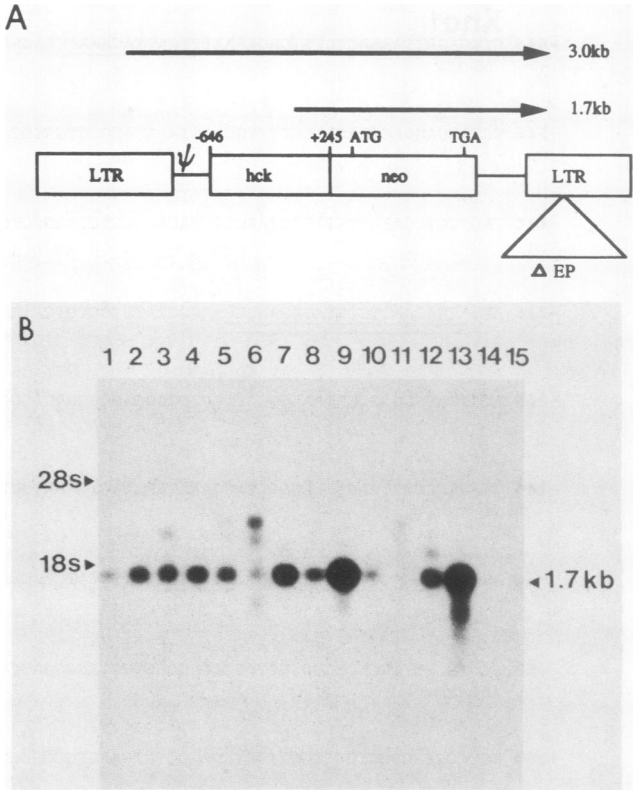

FIG. 3. Functional analysis of the putative *hck* promoter in hematopoietic target cells. (A) Structure of the self-inactivating retroviral vector RV431. The construct shown contains *hck* 5′-flanking sequences (−646 to +245) fused to the bacterial Neo^r gene. The 5′ LTR, the viral packaging signal (ψ), and modified (ΔEP) 3′ LTR are derived from the Moloney murine leukemia virus. Transcripts of 1.7 and 3.0 kb (specified by arrows) would be expected in ψ2 cells transfected with this vector. The 1.7-kb transcript alone is expected in stably infected target cells. (B) Northern hybridization analysis of target cells. Poly(A)$^+$ RNA (1 μg) from G418-resistant FDC-P1 cell lines (lanes 1 through 13) and WEHI-265 cell lines (lanes 14 and 15) was analyzed by using a Neo^r-specific probe and autoradiography.

5′-flanking sequences (Fig. 1, positions −646 to +240) fused to the bacterial Neo^r gene, which confers resistance to G418. Viral sequences, including the 5′ LTR, the viral packaging signal (ψ), and the 3′ LTR from which the viral enhancer and promoter elements have been deleted, were derived from Moloney murine leukemia virus. Cells stably infected with virus express a 1.7-kb transcript, having lost viral promoter and enhancer elements after a single round of replication.

Following cocultivation of virus-producing cell lines with FDC-P1 or WEHI-265 cells, G418-resistant clones arising from the infection were expanded for further analysis. Poly(A)$^+$ RNA was subsequently extracted from individual clones and subjected to Northern analysis with a Neo^r riboprobe (Fig. 3B). Lanes 1 to 13 contain RNA from 13 independently derived FDC-P1 cell lines which harbor the virus with the *hck*-Neo^r fusion gene. Lanes 14 and 15 contain RNA from two WEHI-265-derived cell lines, XW1 and XW2. As expected, each cell line produced a 1.7-kb Neo^r transcript, consistent with initiation of mRNA synthesis from the introduced *hck* promoter (Fig. 3A). The levels of the 1.7-kb Neo^r transcript varied widely among individual cell lines (e.g., compare lanes 1, 4, 7, and 9), suggesting that the site of viral integration can greatly influence the level of

transcription. In certain instances (e.g., lanes 3, 5, and 6), additional hybridizing transcripts were present. Such transcripts may originate from adjacent cellular promoters or may indicate that transcriptional termination within the provirus occurs with variable efficiency.

To examine the fidelity of transcriptional initiation in stably infected FDC-P1 and WEHI-265 cell lines, we analyzed poly(A)$^+$ RNA from parental and virally infected cells by RNase protection (Fig. 4). To distinguish between initiation of endogenous *hck* transcripts and *hck*-Neo^r transcripts, we used riboprobe SBB, transcribed from a plasmid containing the 463-bp *Spe*I-*Bam*HI *hck* genomic fragment (described above) and a contiguous 132-bp *Bam*HI *hck* cDNA fragment (19) (Fig. 4A). Since viral *hck* transcripts contain sequences present only in the genomic fragment, and endogenous transcripts contain sequences found in both cDNA and genomic fragments, we predicted two sets of RNase-protected fragments differing by 132 nt.

The results of the RNase protection analysis are shown in Fig. 4B. Poly(A)$^+$ RNA from PU5-1.8 (lane 2), RAW8 (lane 3), and WEHI-265 cells (lane 4) protects fragments of 320 to 377 nt. The molecular size of these fragments confirms that endogenous *hck* transcripts initiate from multiple start sites within the *hck* gene. Transcripts from three stably infected WEHI-265 cell lines, XW1, XW3, and XW4 (lanes 12 through 14, respectively), initiate at the same sites, although the level of expression is variable. RNA from parental FDC-P1 cells (lane 5) and infected FDC-P1 cell lines F2, F9, F11, and F15 (lanes 7 through 10, respectively) did not protect fragments in this range, reaffirming that the *hck* gene is not expressed in FDC-P1 cells. However, poly(A)$^+$ RNA from stably infected FDC-P1 cell lines F2, F9, F11, and F15 (lanes 7 through 10, respectively) and the WEHI-265 cell lines XW1, XW2, and XW4 (lanes 11 through 13, respectively) protects a series of fragments of 188 to 238 nt, which correspond to transcriptional start sites over a range of 50 nt. The size and heterogeneity of these protected fragments indicate that the *hck* gene and the *hck*-Neo^r fusion gene initiate transcription at essentially the same sites. One class of endogenous *hck* transcript which has no apparent counterpart among virally initiated transcripts is represented by a protected fragment of 377 nt.

RNAs from the two infected FDC-P1 cell lines F2 (lane 7) and F11 (lane 9) protect a single fragment of approximately 460 nt. Such a fragment could arise if the entire *hck* promoter fragment present in the provirus was transcribed into mRNA. This might occur if transcription initiated from a cellular promoter adjacent to the site of viral integration. Collectively, these data show that the putative *hck* gene promoter fragment, defined by the 0.9-kb *Xho*I-*Bam*HI region described above, initiates transcription faithfully in both WEHI-265 cells and FDC-P1 cells.

LPS-induced transcription of *hck* and Neo^r mRNA in stably infected WEHI-265 cells. The levels of *hck* mRNA are transiently elevated in murine bone marrow macrophages (our unpublished data), WEHI-265 cells (our unpublished data), and normal human macrophages (61) in response to LPS. In human macrophages, the steady-state level of *hck* mRNA is elevated eight- to ninefold following exposure to LPS for 6 to 12 h (61). In WEHI-265 cells, a maximum is reached after approximately 8 h (our unpublished data). To determine whether the *hck* promoter region contributes to elevated levels of *hck* mRNA, the stably infected WEHI-265 cell lines XW1, XW2, and XW4 were treated with LPS for 8 h and their RNA was analyzed by RNase protection (Fig. 5). In this analysis the *hck* probe SBB (lane 1), which distinguishes viral and endogenous *hck* transcripts, was combined with a β_2-microglobulin riboprobe (40). The β_2-microglobulin probe, which protects a fragment of 37 nt, was included to enable the amount of RNA in each sample to be quantitated. Poly(A)$^+$ RNA from untreated XW1 (lane 2), XW2 (lane 4), and XW4 cells (lane 6) protected two sets of fragments, of 188 to 238 nt and 320 to 377 nt (size not indicated in Fig. 5). These two sets of fragments represent transcripts initiated at the viral *hck* promoter and the endogenous *hck* promoter, respectively. Clearly, LPS treatment of XW1 (lane 3), XW2 (lane 5), and XW4 cells (lane 7) produced a significant elevation in the steady-state levels of endogenous *hck* and virally derived mRNA.

To quantitate this induction directly, we determined the amount of radioactivity associated with each set of RNase-protected fragments. We did this by subjecting the appropriate pairs of gel slices to scintillation counting. This procedure showed that the level of *hck* mRNA increased 3- to 5-fold following LPS induction, whereas the level of Neo^r RNA increased 1.4- to 2.6-fold in the same interval. These data confirm previous findings that *hck* mRNA expression is elevated in macrophages following exposure to bacterial LPS (61). In addition, the data show that this elevation is mediated at least in part by sequences contained within the *hck* promoter.

DISCUSSION

Our studies have shown that transcription of the murine *hck* gene is initiated from a number of sites spanning a region of 57 nt. Furthermore, the pattern of transcriptional initiation sites in the *hck*-Neo^r reporter gene is essentially identical to that of the endogenous gene, indicating that the selection of initiation sites is dictated by elements contained within the 646-bp flanking sequences of the *hck* gene. In addition, the same start sites are used in cells that lack endogenous *hck* transcripts (e.g., FDC-P1).

The fact that the *hck* promoter is functional in cells which do not express the *hck* gene can be rationalized in terms of the method by which cells containing the fusion gene construct were identified. Only cells which expressed the fusion gene at sufficient levels to survive G418 selection were analyzed. This selection procedure would enrich for cells in which the *hck*-Neo^r virus had integrated into chromosomal regions which favor expression of the provirus. Indeed, the *hck* fusion gene confers G418 resistance to NIH 3T3 cells at a frequency which is only 15% of that of a comparable vector carrying a herpes simplex virus thymidine kinase promoter-Neo^r fusion gene (data not presented). In this regard, it is possible that only a small percentage of integration sites favor the expression of the *hck* fusion gene. Such chromosomal position effects may be sufficient to override any features of the *hck* promoter which may otherwise confer a tissue-specific pattern of expression. Therefore, it is still possible that the *hck* promoter fragment contains elements analogous to those found in the insulin (57), immunoglobulin (2, 13, 15), prolactin (38), and growth hormone (38) genes, which are capable of conferring cell-specific expression on a heterologous gene.

hck transcripts have been shown to accumulate in human macrophages following exposure to LPS (61). This finding is supported by our observation that the murine macrophage-like cell line WEHI-265 responds to LPS with a three- to fivefold elevation in the steady-state level of *hck* mRNA. This increase appears to be partly due to elevated transcriptional activity of the *hck* gene, as initiation of reporter gene

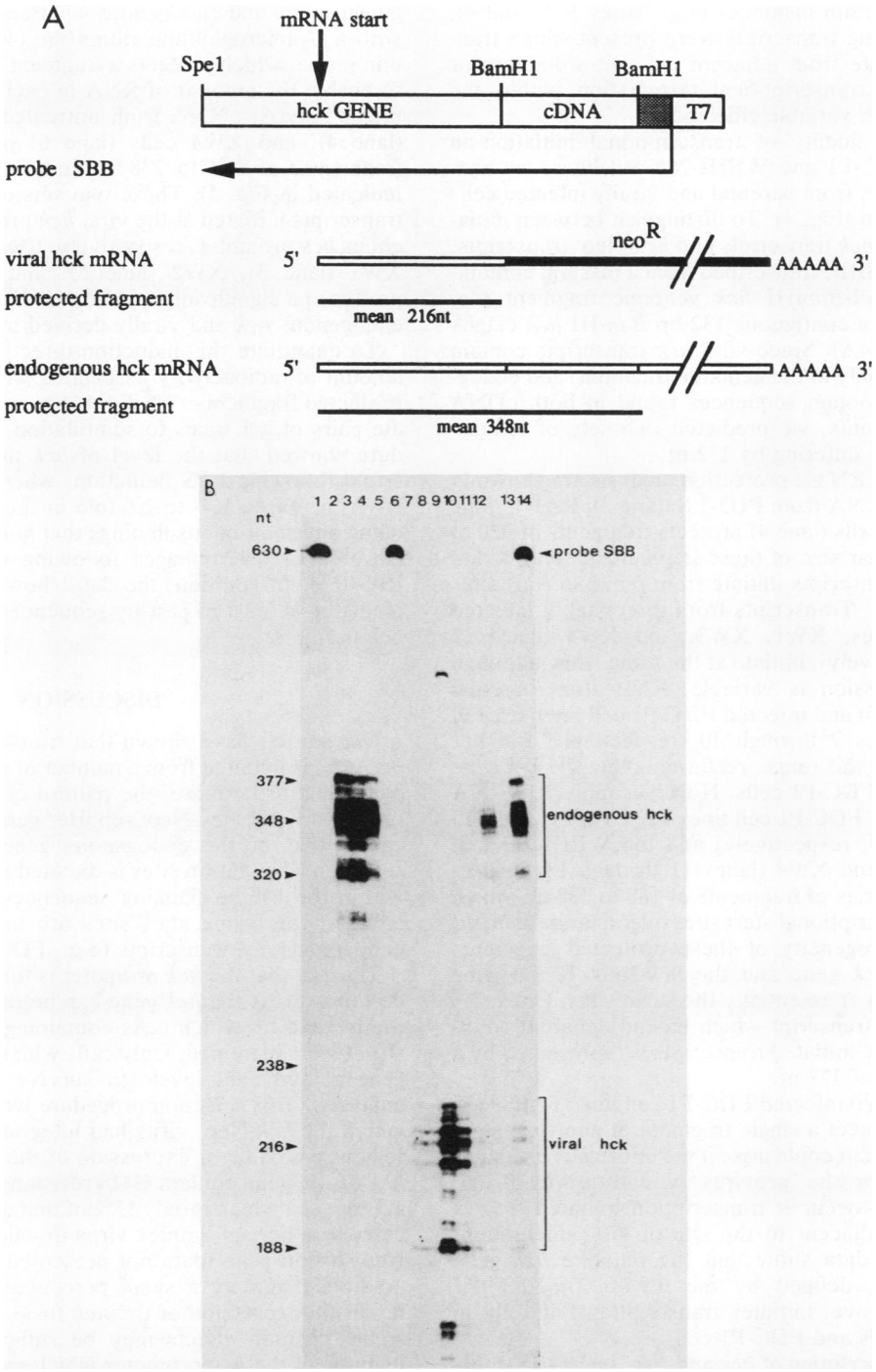

FIG. 4. RNase protection analysis of stably infected hematopoietic cells. (A) Schematic showing the riboprobe SBB and its relationship to the transcriptional start sites of endogenous *hck* and viral *hck* transcripts. The probe was transcribed from a plasmid containing an *SpeI-BamHI* genomic *hck* fragment (Fig. 3) and a contiguous *BamHI hck* cDNA fragment (19). Viral *hck* mRNA is expected to protect fragments of approximately 216 nt, whereas endogenous *hck* mRNA should protect fragments of approximately 348 nt. (B) Electrophoretic analysis of fragments protected from RNase digestion by poly(A)$^+$ RNA (1 μg) from various cell lines: PU5-1.8 (lane 2), RAW8 (lane 3), WEHI-265 (lane 4), FDC-P1 (lane 5), stably infected FDC-P1 cell lines F2, F9, F11, and F15 (lanes 7 through 10, respectively), and stably infected WEHI-265 cell lines XW1, XW2, and XW4 (lanes 11 through 13, respectively). The size and position of the undigested probe (lanes 1, 6, and 14) and the RNase-protected fragments corresponding to endogenous and viral *hck* transcripts are indicated.

transcripts from the *hck* promoter is also elevated under these conditions. Since endogenous *hck* gene expression is elevated to a greater degree than Neo^r expression, the possibility is raised that *cis*-acting elements lying outside the 0.65-kb *hck* 5′-flanking region also contribute to the LPS response. To address this possibility, viral constructs containing 2.5 kb of *hck* 5′-flanking region, including the 0.65-kb fragment analyzed in detail, were introduced into WEHI-265

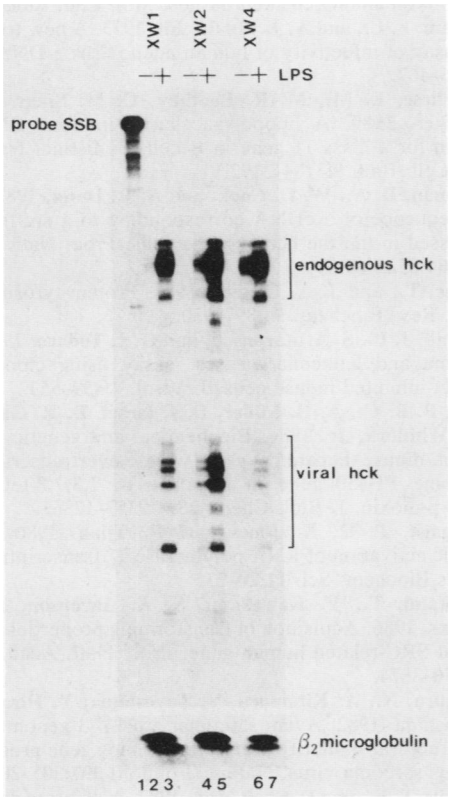

FIG. 5. Elevated levels of endogenous and viral *hck* mRNA in LPS-treated WEHI-265 cell lines. Poly(A)$^+$ RNA (1 μg) from stably infected cell lines XW1, XW2, and XW4, which were cultured in the absence (−) (lanes 2, 4, and 6) or presence (+) (lanes 3, 5, and 7) of 1 μg of LPS per ml for 8 h, was analyzed by RNase protection. The probes used in this analysis were the SBB probe (lane 1), which distinguishes between viral and endogenous *hck* transcripts (Fig. 4), and a β_2-microglobulin riboprobe, which was included to assess the relative amount of poly(A)$^+$ RNA in each sample. Fragments of probe protected by viral and endogenous *hck* transcripts and β_2-microglobulin transcripts are indicated.

cells. RNA from three stably infected cell lines was then analyzed by RNase protection (data not shown). Since we failed to observe any increase in the level of Neo^r mRNA above that measured for the 0.65-kb promoter construct, we assume that distal *cis*-acting elements are required for complete mRNA induction. Alternatively, other mechanisms, such as increased mRNA stability, might contribute to the elevated level of *hck* transcripts observed following treatment of cells with LPS. Indeed, the accumulation of granulocyte-macrophage colony-stimulating factor mRNA in LPS-treated macrophages is largely a posttranscriptional event (52).

A number of genes which are expressed in normal macrophages and macrophage-derived cell lines are known to be regulated by LPS. A sequence within the class II major histocompatibility complex $A_\alpha k$ gene, which is capable of binding a protein found in the nuclei of B cells treated with LPS, was recently identified (18). Examination of the *hck* promoter sequence reveals a 23-bp region which shares significant identity with the LPS-responsive element of the class II $A_\alpha k$ gene (Fig. 6, top). This similarity is particularly interesting since LPS represses the transcription of $A_\alpha k$ in B cells but elevates the transcription of the *hck* gene in macrophages. A motif located at the 3′ end of this 23-bp region shares significant homology with elements in a number of other LPS-responsive genes, including those encoding tumor necrosis factor (37), interleukin-1β (IL-1β) (51), and interleukin-1α (IL-1α) (14). An alignment of these sequences is shown in Fig. 6 (bottom). Dashes were introduced to maximize the alignment. Residues with identity to those found in the $A_\alpha k$ gene are in the large open boxes, and those which differ are indicated by stippled boxes. The DNA elements shown are numbered with respect to the transcriptional start sites of the cognate genes, except for $A_\alpha k$, which

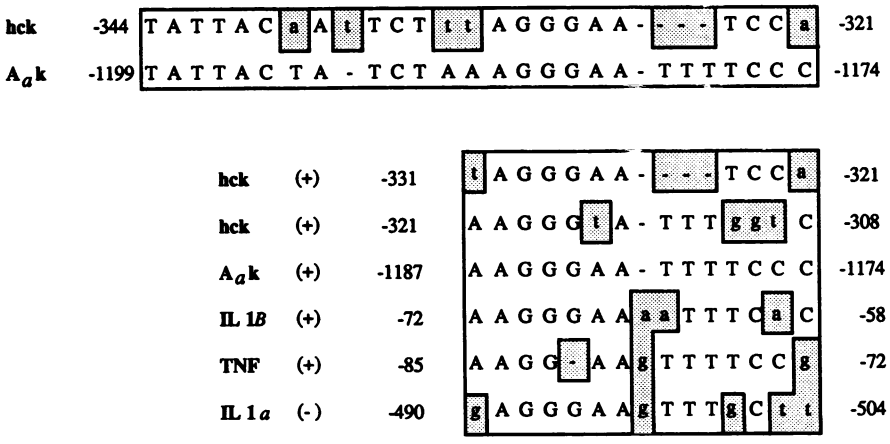

FIG. 6. Alignment of elements from the 5′-flanking regions of genes regulated by LPS. (Top) Alignment between a 23-bp region of the *hck* gene (doubly underlined in Fig. 1) and sequences present in the putative LPS-responsive element of the class II major histocompatibility complex gene $A_\alpha k$ (18). (Bottom) Sequences from the 3′ end of the major histocompatibility complex gene LPS-responsive element are aligned with similar motifs in the 5′-flanking regions of the *hck*, IL-1β, tumor necrosis factor (TNF), and IL-1α genes. The two *hck* motifs are part of an imperfect direct repeat which extends 3′ of the element shown in the upper panel. Single nucleotide breaks in the sequence are indicated by dashes. Bases with identity to the $A_\alpha k$ sequence are represented by capital letters, and those which differ are represented by lowercase letters and highlighted with stippling. Except for IL-1α, for which the nontranscribed (−) strand is shown, all other sequences are derived from the transcribed (+) strand. The sequences are numbered with reference to the start site of transcription (defined as +1), with the exception of $A_\alpha k$, which is numbered from the translational start codon.

is numbered relative to the translational start codon. It is notable that this motif shares a high degree of similarity with the NF-κB binding sequence GGGACTTTCC (47), which is involved in the LPS-mediated transcriptional response of the immunoglobulin κ light-chain gene (48). It is tempting to speculate that the motif identified here is involved in mediating the LPS responsiveness of the *hck* gene. Future studies will focus on identifying regions within the *hck* gene which can bind factors capable of mediating this response. Studies such as this may elucidate the mechanisms by which diverse genes, such as the tumor necrosis factor, IL-1α, IL-1β, and *hck* genes, are regulated by LPS.

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