JOURNAL OF VIROLOGY, Feb. 1997, p. 1237–1245 Vol. 71, No. 2
0022-538X/97/$04.00+0

Shared Promoter Elements between a Viral Superantigen and the Major Histocompatibility Complex Class II-Associated Invariant Chain

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Received 19 July 1996/Accepted 31 October 1996

Superantigens have the ability to stimulate subsets of T lymphocytes bearing particular T-cell receptor Vβ chains. The best-known viral superantigen is Mls, a product of the murine mammary tumor virus (MMTV) *sag* gene. The MMTV superantigen is not displayed by the virus itself; however, after infection of B lymphocytes, the superantigen is expressed. The resulting immune stimulation is essential for viral transmission. We have analyzed the transcriptional elements which control Mls-1 expression. Here we present evidence that a region at the 3′ end of *Mtv-7 env*, Penv2, controls B-cell-specific expression of *sag*. Penv2 has elements homologous with promoters of immunoglobulin H chain, the invariant chain, and major histocompatibility complex class II, suggesting a coordinate regulation of expression of these various B-cell-specific genes and indicating a possible eukaryotic origin of MMTV *sag*. We have determined that both an IgH heptamer element and a Y box are essential for Penv2 promoter activity and that tandem octamer motifs in the U3 region of the 3′ MMTV long terminal repeat function as enhancers. We propose that Penv2 controls constitutive Mls expression in B lymphocytes.

Endogenous murine superantigens were first defined functionally (17) and were later linked to the process of thymic negative selection (30, 38). Several years passed before their genes were identified as part of murine mammary tumor virus (MMTV) proviral DNA, integrated into the germline (4, 19, 64). Interest in superantigens further increased after the discovery that infectious MMTV, a B-type retrovirus which is the causative agent of murine mammary carcinomas, encodes a superantigen (39) and makes use of this molecule for facilitating viral transmission in the host (21).

Superantigens differ from conventional peptide antigens in that they are recognized by T cells in unprocessed form (13, 18); therefore, presentation is major histocompatibility complex (MHC) class II dependent but not restricted. The association of superantigens with the T-cell receptor (TCR) depends only on the Vβ region for recognition (62), rendering 5 to 10% of primary T cells available to mount a response to any superantigen, and they elicit an unusually strong primary response.

The relevance of the superantigen for the completion of the life cycle of infectious MMTV is now clear. This retrovirus is transmitted vertically through milk from mother to offspring. From the work of Golovkina and colleagues we know that superantigen-reactive T cells are required for the transmission of MMTV from the primary residence in the gut of the suckling newborn to the final destination in mammary tissue (21). It is only here that MMTV replicates spontaneously, due to the glucocorticoid response elements in the viral long terminal repeat (LTR), potentially resulting in mammary carcinomas. Replication is enhanced during lactation, and the incidence of mammary tumors increases significantly with each pregnancy. In contrast, viral replication in any other somatic tissue is minimal, and superantigen-induced immune stimulation is required for the transmission of this retrovirus. It is well established that successive immunological reactions take place, subsequent to infection of the B cell (5). Infected B cells transcribe the MMTV superantigen and present it on the cell membrane to T cells in the context of MHC class II. Superantigen-reactive T cells, expressing the relevant TCR Vβ chain (24, 26), in turn stimulate the MMTV-infected B cells by producing growth factors and providing accessory molecules. Since cellular activation is generally required for retroviral replication, this stimulation leads to amplification of the virus, and it is infected B cells which finally transport the virus to the mammary tissue (1).

Understanding the control of superantigen expression in B cells is important, as this molecule is essential to the viral life cycle. Three promoters have previously been reported to play a role in the expression of the MMTV superantigen: the classical ^{MMTV}P1 in the U5 region of the 5′ LTR, from which all MMTV transcripts originate (8, 44); ^{MMTV}P2, a promoter mapping to the U3 region of the 5′ LTR (22); and Penv, a T-cell-specific phorbol myristate acetate-inducible promoter encoded within the 5′ end of the *env* gene (41). Here we present evidence of a new promoter, Penv2, mapping to the 3′ region of the *env* gene of endogenous *Mtv-7* and infectious JYG MMTV (67). Penv2 contains elements that are similar to the promoters of MHC class II (20), the MHC class II-associated invariant chain (Ii) (6), and, most likely, H-2M (10), genes that share B-cell-specific regulation of expression. An increase in class II expression has been associated with an augmentation of the superantigen response, but no direct link to an increase in *sag* transcription has been established so far (11, 35). We predict that upregulation of transcription factors that bind to MHC class II promoters might be capable of simultaneously activating *sag* transcription.

The similarities in promoter elements and protein structures (27a) between the MMTV *sag* and the Ii genes and their products, respectively, point to a possible eukaryotic origin of *sag*. It is well documented that retroviruses have the capacity to

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incorporate and maintain host genes, if they provide a selective advantage for the virus.

MATERIALS AND METHODS

Cell lines. LBB.A, an Mls-1-expressing *Mtv-7*$^+$ B-cell hybridoma cell line derived from a fusion of BALB/c and RF/J B cells, and its Mls-1$^-$ *Mtv-7*$^-$ variant LBB.11 were previously described (4, 46). A20 is a BALB/c-derived B-cell lymphoma cell line expressing surface immunoglobulin G (IgG) (TIB 208; obtained from the American Type Culture Collection [ATCC], Rockville, Md.) (31). MPC11 is a γ2b/κ-producing myeloma cell line derived from a BALB/c tumor (gift of L. A. Eckhardt, Hunter College, New York, N.Y.) (33). The human Epstein-Barr virus-transformed B-lymphoblastoid cell line (B-LCL) 8.1.6 was used as an Mls-negative control for primer extension studies (40). BW5147 .G.1.4.OUA^R.1 is a variant subline of the AKR/J mouse thymus-derived cell line BW5147 (ATCC, CRL1588) (28). EL4.IL-2 (ATCC, TIB 181) is a variant subline of the C57BL/6 mouse lymphoma cell line EL4 (16). The following murine fibroblast cell lines were used: L cells (ATCC); DAP-3 cells, a subline of L cells which has increased Ii chain expression (34); DAP-3 cells stably expressing HLA.DR1 (27) (DAP-3 lines were gifts of R. N. Germain, National Institutes of Health [NIH], Bethesda, Md.); and NIH 3T3, an NIH Swiss mouse-derived line (ATCC, CRL1658) (29). The JYG mammary tumor cell line which produces JYG MMTV (obtained from N. H. Sarkar, Medical College of Georgia, Augusta) (53) was used. All cells were maintained in RPMI 1640 (Gibco BRL, Gaithersburg, Md.) supplemented with 10 to 15% fetal calf serum (FCS; Intergen, Purchase, N.Y.), 50 μM 2-mercaptoethanol (2-ME), nonessential (NE) amino acids, 1 mM sodium pyruvate, 100 μg of penicillin/ml, and 100 U of streptomycin/ml, referred to as complete RPMI.

Plasmids and genetic modifications. The first group of constructs were created by replacing MMTV *sag* with the *luciferase* gene in plasmids previously tested functionally for *sag* expression (4). The *luciferase* gene cassette originated from a *Bgl*II-*Dra*I fragment of the vector pGL2-Basic (Promega, Madison, Wis.) and was cloned into pII-11dH. This vector, pII-11dH, encodes the *env* gene and the 3′ LTR of *Mtv-7* as an *Eco*RI-*Hin*dIII fragment, derived from a previously described construct, pII-11 (4). Construct pELO contains a pII-11dH *Bgl*II fragment of 2,053 bp encoding *Mtv-7 env*, which was cloned into the *Bgl*II site of pGL2-Basic in the same orientation as that of the *luciferase* gene. This *env* fragment maps between positions 6604 and 8657 of the BR6 provirus sequence (GenBank accession number, M15122) (42). Construct pELO also contains a fragment of the *Mtv-7* 3′ LTR-U3 region that encodes enhancer elements (23) and that was PCR cloned into the *Bam*HI and *Sal*I sites downstream of *luciferase*. Primers used for PCR were as follows: 5′ CGGGATCCCAGGTCTACTTGCG GTTC 3′ (*Bam*HI site underlined) and 5′ ACGCGTCGACTCATGATTTACA TAAGCA 3′ (*Sal*I site underlined). The above-described fragment corresponds to the region on the BR6 provirus sequence between positions 9669 and 9861 (42). To create pELO600, the *Bgl*II *env* fragment in pELO was replaced by a 600-bp fragment, obtained after nested deletions of the 5′ end of the *env* gene (see Fig. 1). pΔOct600 is a derivative of pELO600 with a deletion of the 3′ LTR-U3 region fragment, i.e., from *Bam*HI to *Sal*I. Construct pmOct600 was derived from pELO600 after site-directed mutagenesis (Clontech, Palo Alto, Calif.) to mutate the octamer motifs which are encoded in the U3 enhancer region described above. The primer used for site-directed mutagenesis of octamers was as follows: 5′ TATCCAAGTCTTCTGAACATGCTTCTGATCAT CATGAGTCGAC 3′. The above-described mutations were previously shown to reduce binding of Oct proteins (36). Construct pEnvA100 was obtained after subcloning a *Bgl*II-*Ssp*I fragment from pELO600 (see Fig. 3B, between positions 1 and 110) into construct pELO, previously digested with *Sst*I and *Bgl*II to remove the 2,053-bp *env* gene fragment. pEnvD100 was obtained after subcloning a *Hpa*I-*Dra*III fragment from pELO600 (see Fig. 3B, between positions 220 and 360) into construct pELO, previously digested with *Sst*I and *Bgl*II as described above. For the Penv2 promoter deletion experiments (see Fig. 5), the following PCR primers were used: Env1, 5′ GGAGATCTATTTCATTTTTAT AGG 3′; Env2, 5′ GAAGATCTCCCTGCTTTTGGTCA 3′; Env3, 5′ GCAGA TCTGATTTTCCCCATTGT 3′; Env4, 5′ CCGAAGCTTGATCTGACTGCAC TTG 3′; and Env5, 5′ CCGAAGCTTCTTCGCAAAGCACTG 3′. Construct pEnvA100 was used as the PCR template. PCR fragments were cloned into construct pELO, which had been previously digested with *Bgl*II and *Hin*dIII to remove the *env* gene 2,053-bp fragment. To create the deletion constructs shown in Fig. 6, the above-listed primers were paired as follows: Env1 and Env5 for construct 3; Env2 and Env4 for construct 2; and Env3 and Env4 for construct 4. For Penv2 promoter site-directed mutagenesis, the following primers were used to mutate the specified promoter elements: 5′ GATTTTCCATATGCTTTTCC AGTGC 3′, Y box (65); 5′ GTCATTGTACAGCTGATTTTCCCC 3′, IgH heptamer (IgHep or I) box (32); and 5′ CTGCTTTTGTGCACTGTACTTATG 3′, HP1 (H) box (52). A second set of primers was used for the site-directed mutagenesis selection step (Clontech): 5′ GCAGCCACTAGTAACAGGATT 3′ (*Alw*NI→*Spe*I), and 5′ GCAGCCACTGGTAACAGGATT 3′ (*Spe*I→*Alw*NI). The deletion and site-directed mutagenesis constructs were sequenced for verification. Sequence analyses to identify promoter elements were performed with Genetics Computer Group data banks.

Transfections and luciferase assays. All plasmids were isolated with Qiagen (Chatsworth, Calif.)-tip columns. A sample plasmid (12 μg) was coprecipitated with 10 μg of pMSVβ-gal per transfection. Plasmids were washed two times with 70% ethyl alcohol and resuspended in 50 μl of 0.1× Tris-EDTA. For electroporation, a previously described transfection protocol was slightly modified (55). Cultures were split the day before use to bring them to a density of 10^6 cells/ml at harvest. Cells were washed twice and resuspended at 1.8×10^7/ml in RPMI 1640 supplemented with 1% FCS, 50 μM 2-ME, NE amino acids, and 1 mM sodium pyruvate. We used a volume of 500 μl of resuspended cells/0.4-cm-electrode-gap cuvette (Bio-Rad, Hercules, Calif.). All electroporations were carried out at room temperature with a Gene Pulser (Bio-Rad) with a capacitance extender. LBB.A, LBB.11, A20, and BW5147 cells were electroporated at 320 V and 960 μF, and MPC11 cells were electroporated at 300 V and 960 μF. Cuvettes were transferred to an ice bath for 10 min after electroporation. Samples were then gently transferred to six-well plates (Falcon, Lincoln Park, N.J.) and diluted to a final volume of 7 ml with complete RPMI. Cells were harvested for assay 18 to 24 h after electroporation. For EL4.IL-2, the protocol had the following modifications: 25 μg of sample plasmid, combined with 10 μg of pMSVβ-gal, was used per transfection; cells were resuspended at 4×10^7/ml and electroporated at 450 V and 125 μF. Fibroblasts and JYG mammary tumor cell lines were transfected by the DEAE-dextran–chloroquine diphosphate method (54). Cells were grown in 100-mm-diameter petri dishes to 80% confluency. The sample plasmid (12 μg) was coprecipitated with 10 μg of pMSVβ-gal per transfection and suspended in a final volume of 50 μl of 0.1× Tris-EDTA. Each plasmid suspension was diluted in 5 ml of RPMI 1640 supplemented with 5% FCS, 50 μM 2-ME, NE amino acids, and 1 mM sodium pyruvate; 200 μl of 25× DEAE-dextran–chloroquine diphosphate was added before each suspension was applied to a phosphate-buffered saline-washed cell monolayer. Cells were incubated at 37°C; DAP-3 and L cells were incubated for 90 min and NIH 3T3 and JYG tumor cells were incubated for 60 min. After the incubation period, supernatants were aspirated and cells were shocked with phosphate-buffered saline–10% dimethyl sulfoxide for 2 min. Cells were then incubated at 37°C with 10 ml of complete RPMI and harvested for assay after 48 h. For luciferase assays, we used the Promega system and protocol (TB101). Cells were lysed with 200 μl of 1× lysis reagent. Luciferase activity of a 20-μl aliquot was measured for 10 s in an ILA911 luminometer (Tropix, Bedford, Mass.). Concomitant β-galactosidase expression from the cotransfected control, pMSVβ-gal, was measured to normalize samples; activities of 50-μl aliquots were detected by the LumiGAL system (Clontech) and the manufacturer's chemiluminescent β-galactosidase assay protocol.

RNA isolation and primer extension. Total RNA was isolated with Trizol reagent by the Gibco BRL protocol. RNA from transiently transfected cells was isolated 24 h after transfection. Splenocytes were isolated from an AKD2/J F1 mouse and were stimulated with lipopolysaccharide (Sigma, St. Louis, Mo.) at a final concentration of 50 μg/ml in RPMI 1640 for 48 h before total RNA isolation. For primer extension, mRNA was isolated from total RNA preparations by the PolyATtract mRNA isolation system III (Promega). For primer extensions, 100 ng of primer was labelled with 450 μCi of [γ-^{32}P]ATP by using 20 U of polynucleotide kinase (New England BioLabs, Beverly, Mass.) in a 30-μl reaction mixture, incubated at 37°C for 1 h. Free nucleotides were removed with a BioSpin column 6 (Bio-Rad). Labelled primer hybridizations to RNA and extension reactions were carried out as previously described, with some modifications (2). For hybridization, tubes were submerged for 5 min in a 55°C bath and then transferred to a 37°C bath and incubated overnight. Extension reactions were performed with 200 U of SuperScript II RNase H$^-$ reverse transcriptase (Gibco BRL), 40 U of recombinant RNasin (Promega), and 5× First Strand Buffer (Gibco BRL). Actinomycin D was not used. Reactions were carried out at 47°C for 1 h and stopped as described previously. Primers used were 5′ GAGG ATAGAATGGCGCCGGGCC 3′ for *luciferase*; 5′ GGCTGCTTCTCTCCTAA GTGTAG 3′ for *sag*; and 5′ GCGCCCAATACGGCCAAATCCGTTC 3′ for murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For *luciferase* reporter primer extension, 15 and 30 μg of total RNA were used; for *sag* primer extensions, 5 μg of mRNA was used; and for GAPDH primer extension, 250 ng of mRNA was used. All sequencing reactions were performed with a U.S. Biochemicals (Cleveland, Ohio) Sequenase kit (version 2.0) by following the U.S. Biochemicals alkaline-denaturation method.

RESULTS

Deletion analysis reveals a promoter region mapping to the 3′ end of the MMTV *env* gene. Previous work from our laboratory suggested that transcriptional control elements are encoded within the *env* gene of *Mtv-7*, leading to increased levels of *sag* expression in B cells, as determined by functional T-cell proliferation assays (reference 4 and unpublished observations). To further analyze these data, we replaced the *sag* gene with a *luciferase* gene cassette (see the map in Fig. 1A). Transient and stable transfections into LBB.11, a B-cell hybridoma cell line, showed significant luciferase activity. To map the novel promoter region, deletional analyses of the *env* gene were carried out. First, we subcloned an *Mtv-7* fragment en-

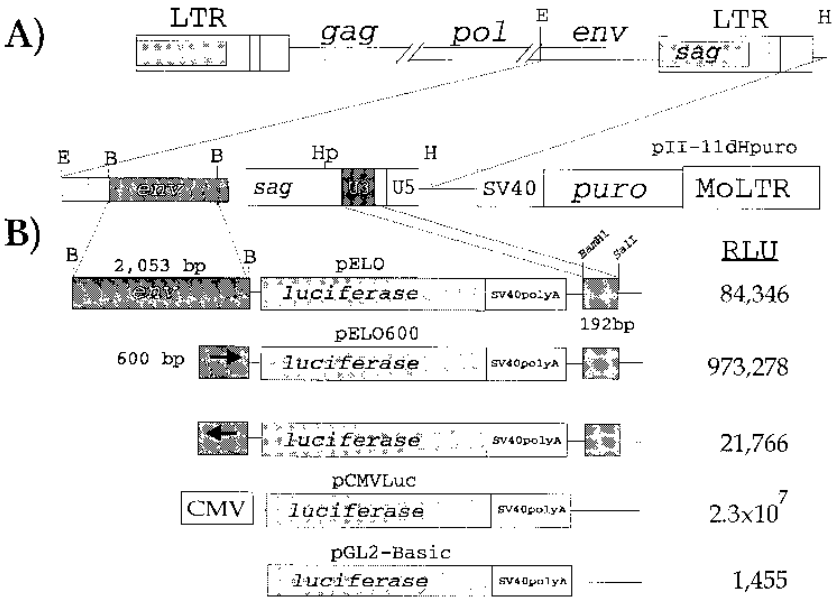

FIG. 1. Diagrams of MMTV and luciferase constructs. (A) The *Eco*RI-*Hin*dIII fragment of *Mtv-7* was subcloned into the pII-11dHpuro plasmid. The *Bgl*II-*Hpa*I fragment encoding *sag* was replaced by a luciferase cassette. (B) The 2,053-bp *Bgl*II fragment, encoding most of the *env* gene, was subcloned into the multiple cloning site of pGL2-Basic. The 192-bp *Bam*HI-*Sal*I fragment from the 3′ LTR U3 region was subcloned 3′ of the luciferase gene, resulting in construct pELO. Construct pELO600 was obtained by nested deletions, starting at the 5′ end of the *env* gene. Luciferase activity after transfection of LBB.11 B cells was measured as relative light units (RLU). The positive control was pCMVLuc, and the negative control was pGL2-Basic. E, *Eco*RI; H, *Hin*dIII; B, *Bgl*II; Hp, *Hpa*I; SV40, simian virus 40.

coding a large portion of the *env* precursor (from a *Bgl*II site 36 bp upstream of the 3′ LTR [4] to a second *Bgl*II site 2,053 bp upstream of the 3′ LTR) into the pGL2-Basic vector (Fig. 1B). Second, we subcloned a 192-bp fragment which maps 3′ of the *sag* gene in the U3 region of the LTR and encodes enhancer elements potentially relevant in B-cell expression, including two tandem octamer motifs which are located upstream of the TATA box (the TATA box was excluded) (7, 23). We compared the promoter activity of this construct, pELO (Fig. 1B), to those of the various 5′ deletion constructs and observed that a 600-bp 3′ *env* fragment was sufficient to obtain maximal transcriptional effect after transient transfection of the LBB.11 cell line. A substantial increase in activity is seen with the pELO600 construct compared to the activity of the pELO construct, suggesting the presence of a negative element in the 5′ portion of the *env* gene.

We studied the tissue specificity of this novel promoter region by transiently transfecting various cell types (Fig. 2A). Luciferase activity was significantly higher in the B-cell hybridoma cell line LBB.11, compared to that of the plasmacytoma cell line MPC11 or those of the fibroblast cell lines DAP-3 and NIH 3T3. No reporter activity was detected in the T-cell lines BW5147 and EL-4 (data not shown). Furthermore, when LBB.11 cells were transfected with a linearized construct that contained the 3′ U3 region fragment, pELO600, a twofold increase in luciferase activity was seen compared to the activity obtained in the absence of this segment, i.e., when LBB.11 cells were transfected with pΔOct600 ($P < 0.02$). To investigate the possible role of the U3 region tandem octamer motifs in this B-cell-specific enhancement, site-directed mutations were made in the octamer sequences and tested in B cells and fibroblasts. As can be seen in Fig. 2B, in the LBB.11 B-cell hybridoma cell line, the mutant plasmid pmOct600 yielded a 50% reduction of the luciferase activity seen with the linearized wild-type construct pELO600 ($P < 0.001$). On the other hand, less reduction was observed in DAP-3 fibroblasts ($P < 0.01$). These results suggest that the octamer motifs in the 3′ LTR of *Mtv-7* confer a B-cell-specific enhancement in the context of the newly defined *env* promoter.

The *env* promoter with maximal activity in B-cell lines maps to a 100-bp region. In order to map the transcriptional start site of this novel *env* promoter, we carried out primer extension analyses. We used a luciferase-specific primer to avoid confusion with the various endogenous MMTV transcripts that contain the *sag* gene. Figure 3 summarizes the results obtained after transient transfection of LBB.11 cells with the pELO600 construct. The sequence shows the location of the identified start sites (Fig. 3A) in relation to the *Bgl*II site in the 3′ end of the *env* gene, arbitrarily assigned to position 1 (Fig. 3B). Identical results were obtained when primer extension was performed in LBB.11 cells that were stably transfected with pELO, confirming that the identified start sites were not an artifact due to transient expression (data not shown). As can be seen in Fig. 3A, start site A is the predominant band, appearing as a doublet. Start sites B and C are intermediate, and start site D is weakest and could be detected only after prolonged exposure of the X-ray film. Sequence analysis revealed a TATA box and an octamer motif 24 and 60 bp, respectively, upstream of start site D (Fig. 3B). The regions surrounding start sites A and D were subcloned into pGL2-Basic, resulting in constructs pEnvA100 and pEnvD100, respectively, and tested for promoter activity by transient transfection of LBB.11 cells (Fig. 3C). Luciferase activity was detected only with the A start site construct in the sense orientation, implying that this region encodes the promoter. To further substantiate this finding, transient transfections were performed with the same cell types used for Fig. 2 and the pELO600 construct was compared to the pEnvA100 construct. These experiments revealed that the activity obtained in all cell lines with construct pEnv A100 was equivalent to that obtained with the pELO600 construct, indicating that start site B, C, or D does not significantly contribute to promoter activity (data not shown). Thus, we conclude that

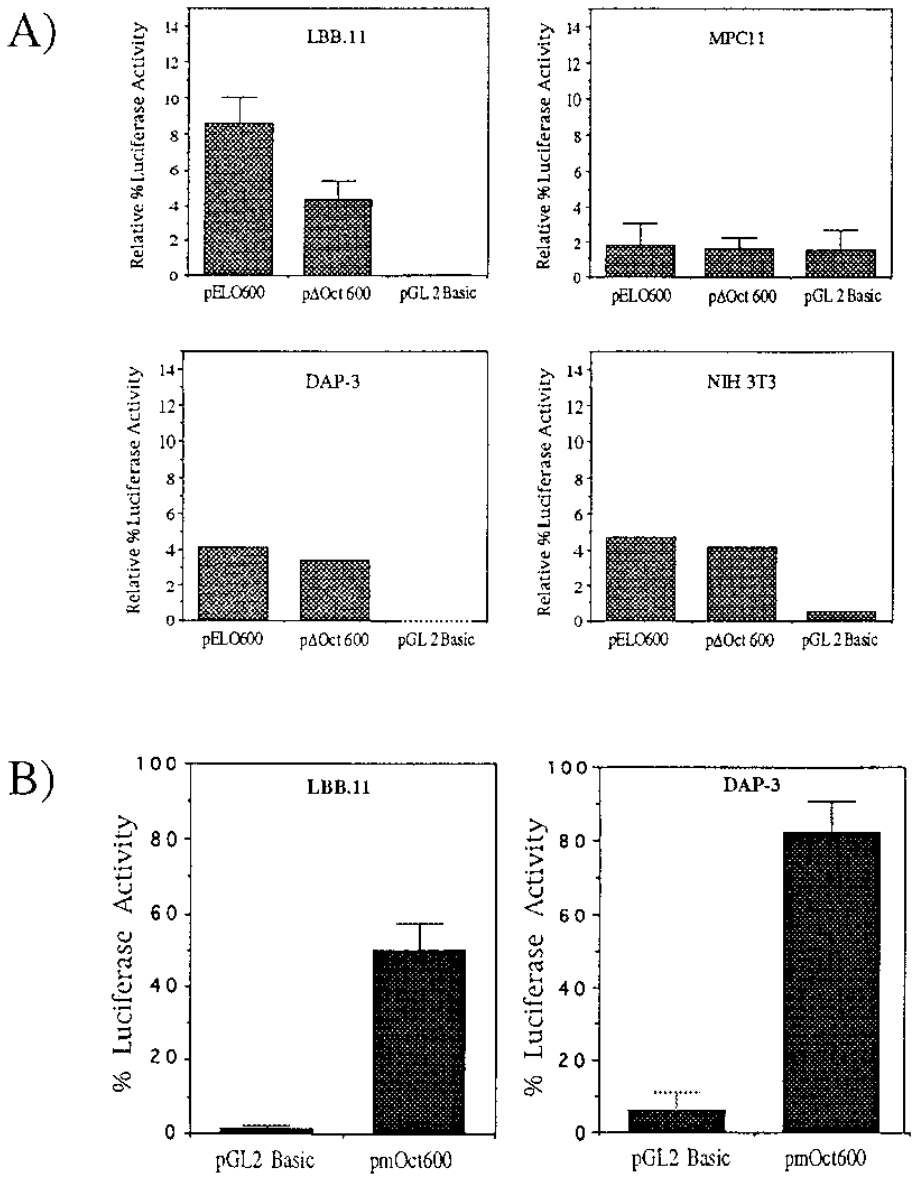

FIG. 2. Tissue specificity of the *env* promoter and enhancement due to the downstream U3 region elements. All plasmids were linearized with *Sal*I prior to transfection. (A) Graphs show relative percentages of luciferase activity compared to those obtained with the CMVLuc construct (100%), determined by the formula (sample RLU/pCMVLuc RLU) × 100, where RLU is relative light units. For LBB.11 and MPC11, the average of three experiments is shown. For DAP-3 and NIH 3T3, representative experimental results from a total of two experiments are shown. pΔOct600 does not contain the 3′ U3 region fragment. (B) The pmOct600 construct has a mutation in the tandem octamer sites in the 3′ U3 region fragment (see Materials and Methods). Graphs show percentages of luciferase activity obtained with the wild-type pELO600 construct, as determined by the formula (sample RLU/pELO600 RLU) × 100. The averages of five and four experiments are shown for LBB.11 and DAP-3, respectively.

the 110-bp A region is sufficient for maximal expression. From all the cell types tested, promoter activity was highest in the B-cell hybridoma cell line LBB.11, which showed significantly greater luciferase activity than the B-cell lymphoma cell line A20. Interestingly, this parallels the expression pattern seen with the endogenous MMTV *sag* products; namely, LBB.11 is vastly superior to A20 in expressing MMTV superantigens encoded by identical viral strains, as judged by LBB.11's capacity to stimulate the appropriate T-cell subsets, while no difference is seen between these two B-cell lymphoma cell lines in presenting conventional peptide antigen (4a). Thus, the difference in superantigen presentation cannot be due to differential MHC class II expression but must reflect the actual level of MMTV *sag* product. In addition, the promoter activity is higher in DAP-3 than in NIH 3T3 fibroblasts. These two lines differ in their abilities to express the class II-associated Ii; only DAP-3 is Ii^+. The potential relevance of this finding in the function of the novel *env* promoter will be discussed below.

Sequence analysis of the A region revealed imperfect S and Y boxes which are highly homologous to both human and murine Ii and to MHC class II promoter elements (10) (Fig. 4). The relevance of these *cis*-acting promoter elements in the expression of Ii and MHC class II genes has been extensively studied (reviewed by Glimcher and Kara [20]). The Y box element binds the factor NF-Y and functions to augment X-box-directed expression, while the S box (also referred to as

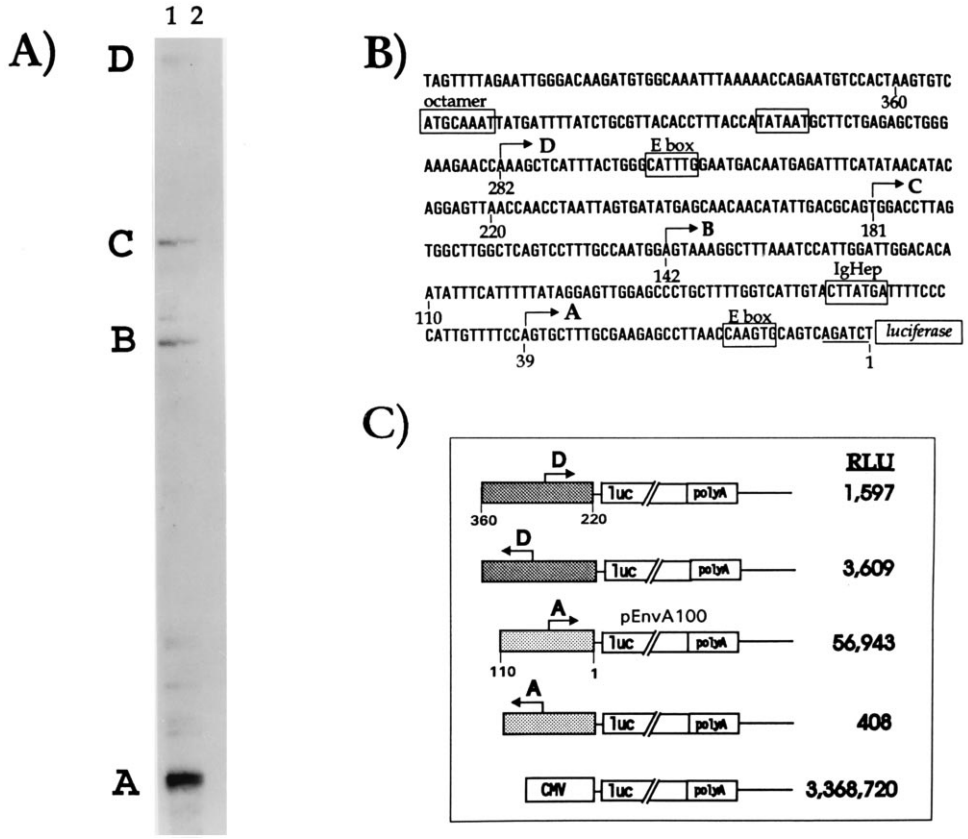

FIG. 3. Mapping of the *env* promoter start site. (A) Primer extension analyses were carried out with a luciferase-specific primer. The autoradiograph shows the results obtained with 30 μg (lane 1) or 15 μg (lane 2) of total RNA from LBB.11 cells, harvested 24 h after transfection with pELO600. Potential start sites are designated A to D. (B) Nucleotide sequence of the *env* fragment containing the four potential start sites. The start sites are marked by arrows. Transcriptional control elements are indicated by boxes. The *Bgl*II site is underlined. These sequence data are available from GenBank under accession number BankIt82110 U79749. (C) Results of luciferase assays after subcloning region D, from positions 282 to 360 (see the sequence in panel B), and region A, from positions 1 to 110, into pGL2-Basic in both orientations. RLU, relative light units; CMV, cytomegalovirus promoter.

the W box) enhances activity in B cells and is required for gamma interferon (IFN-γ) induction (50). In addition, an HP1 (H) consensus sequence, an imperfect IgHep (I), an IFN-γ-activated site (GAS) element, and an E box were identified in this region. The HP1 element has been associated with liver-specific expression of several genes (52). IgHep belongs to the sequences required for the full, lymph tissue-specific activity of the Ig promoter (32). The GAS, which is inverted in this MMTV promoter, is identical to the GAS in the *ICSBP* gene (12). The E box is an element of a TATA-less promoter, shown to be crucial for the expression of the *CD2* gene during T-cell development (47).

Deletional and site-directed mutagenesis analyses of the *cis*-acting promoter elements in region A. To determine the relevance of the promoter elements predicted by sequence analysis, deletional constructs were made by PCR. The Y box was considered a prime candidate for promoter function, based on the results obtained in mutational studies of the class II (58) and Ii (6) gene promoters; thus, the Y box was altered by site-directed mutagenesis. Figure 5 summarizes the results obtained after transient transfection of these constructs into LBB.11 B-cell hybridoma cells. The absence of a functional Y box (construct 1b) leads to a 70% reduction in luciferase activity, as compared to that of the wild-type construct (construct 1a). Deletion of the S box (construct 2a) reduces activity less than twofold, which correlates with the enhancer function attributed to this element in the expression of MHC class II and Ii genes (3, 58). Simultaneous deletion of the GAS and E box sequences reduced activity almost twofold (construct 3a); similarly, deletion of the S, H, and I elements (construct 4a) resulted in a minimal promoter with a twofold reduction of activity. Adding the Y box mutation to this construct (construct 4b) yielded an almost 10-fold reduction. These experiments clearly define the relevance of the Y box in promoter function.

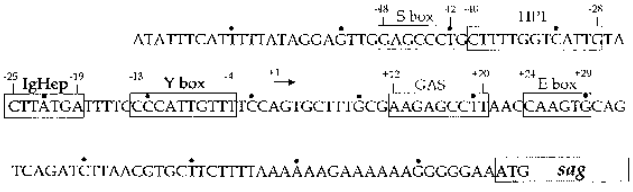

FIG. 4. DNA sequence of the A start site in the *Mtv-7 env* region. Promoter elements identified by sequence analysis are shown in boxes, and positions relative to the A start site (+1 arrow) are indicated.

Previous reports of NF-Y, the ubiquitous Y box binding factor, have suggested that this factor is a crucial transcriptional activator which functions through cooperative binding to other promoter elements (66). In an attempt to determine whether this cooperative binding of NF-Y is effective in the MMTV *env* promoter, we carried out site-directed mutations

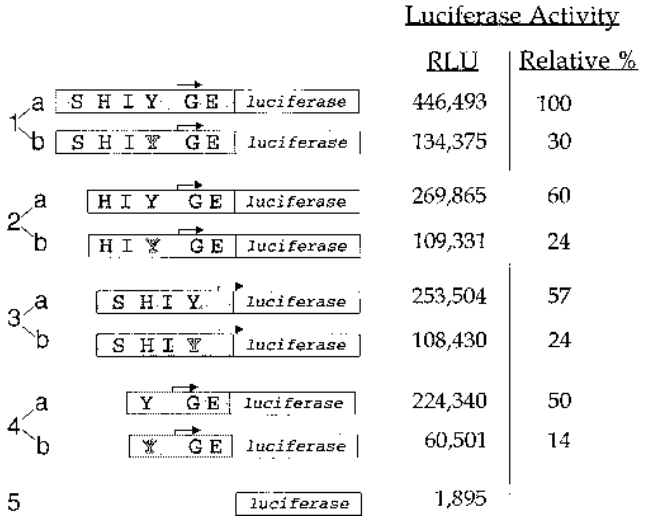

FIG. 5. Deletion and site-directed mutagenesis analyses of the A region promoter elements. Letters: S, S box; H, HP1; I, IgHep; Y, Y box; G, GAS element; and E, E box. The start site (+1) is indicated by an arrow. Deletion of elements is indicated by the absence of respective letters. Mutations are indicated in open letters. One result representative of three experiments is shown. Luciferase activity is shown in relative light units (RLU) as well as in relative percentages of the activity of the pEnvA100 construct, as determined by the formula (sample RLU/construct 1a RLU) × 100.

of the elements upstream of the Y box (Fig. 6). While mutation of the H (construct 2) or I (construct 3) box reduced activity by about 50% in B cells and fibroblasts, respectively, the double mutation in the I and Y elements (construct 6) showed a 10-fold reduction in activity, as measured after transient transfection of LBB.11 B cells as well as DAP-3 fibroblasts. This double mutation in the I and Y elements resulted in equivalent losses of promoter activity in A20 B-cell lymphoma and JYG mammary tumor cell lines (data not shown).

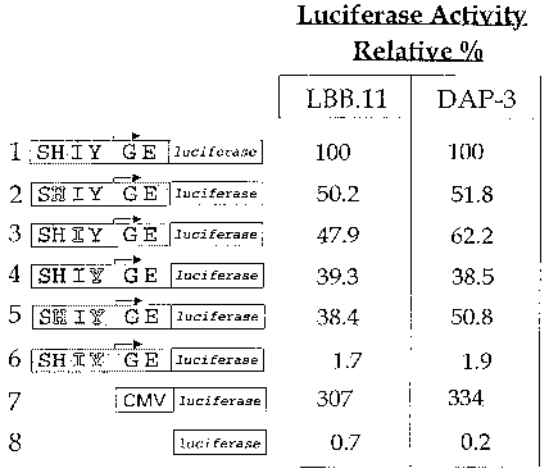

FIG. 6. Analyses of the A region promoter elements by site-directed mutagenesis. Mutations are indicated by open letters. Letters: S, S box; H, H box; I, I box; Y, Y box; G, GAS element; and E, E box. The start site (+1) is indicated by an arrow. A result representative of three experiments is shown. Relative percentages of the luciferase activity obtained with the pEnvA100 construct were calculated by the formula (sample RLU/construct 1 RLU) × 100. Relative light units obtained with construct 1 (pEnvA100) were 1,005,461 for LBB.11 and 2,388,352 for DAP-3. CMV, cytomegalovirus promoter.

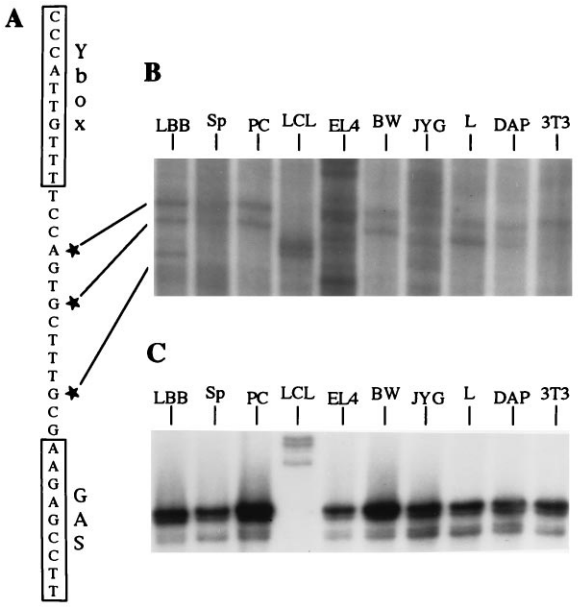

FIG. 7. Primer extension analysis for the identification of the *sag* message originating at start site A. (A) The DNA sequence between the Y box and the GAS element is shown, and the transcription start sites are indicated by stars. (B) Autoradiograph of the primer extension reaction with a *sag*-specific primer. (C) Autoradiograph of the primer extension reaction with a GAPDH-specific primer. Lane designations are as follows: LBB, LBB.A cells; Sp, splenocytes; PC, MPC11 plasmacytoma cells; LCL, B-LCL; EL4, EL4 T cells; BW, BW5147 T cells; JYG, JYG MMTV tumor cells; L, L-cell fibroblasts; DAP, DAP-3 (DR1$^+$) fibroblasts; and 3T3, NIH 3T3 fibroblasts.

A superantigen message corresponding to the novel promoter is present in murine cell lines. We proceeded to search for a *sag* message in murine cell lines corresponding to start site A, as identified in Fig. 3. Initially, we attempted RNase protection assays; however, the results were obscured by various messages obtained from the location of the predicted start site overlapping the splice acceptor site, SA_{orf} (between −7 and +2 in Fig. 4), previously described for two other *sag* messages that originate in ^{MMTV}P2 or ^{MMTV}P1 (22). In addition, the abundant *env* message encodes the A start site region. However, using primer extension with an oligonucleotide mapping to the 5′ end of *Mtv-7 sag*, we were able to identify in LBB.A, an *Mtv-7*$^+$ B-cell lymphoma cell line, the initiation site corresponding to start site A (the most 5′ start site in Fig. 7A). Two other start sites further downstream were also detected. The two more 5′ start sites were identified in splenocytes and in MPC11, BW5147, and fibroblast cell lines, while in NIH 3T3 fibroblasts we identified only the most 5′ start site. None of these start sites were detectable in EL4 T-cell lymphoma or JYG mammary tumor cells or in the human B-LCL. Primer extension reactions with a GAPDH 5′ primer were conducted with an aliquot of each sample to normalize the relative mRNA concentrations (Fig. 7B). The results obtained with these experiments suggest that this novel *sag* message is of extremely low abundance in murine cell lines at basal transcription level.

DISCUSSION

The control of MMTV *sag* transcription is not well understood, despite extensive work in this area (11, 23, 68). The functional data suggest that this molecule is tightly controlled, both in terms of tissue specificity and level of expression. Three

promoters linked to *sag* expression have been described so far; P1, the classical promoter of MMTV, located at the U3 region boundary and used for the expression of the retroviral genome and structural genes (8, 44); P2, a promoter encoded within the U3 region of the 5′ LTR (22); and Penv, a phorbol myristate acetate-inducible promoter encoded within the 5′ end of the *env* gene (41). Here we provide evidence of a new promoter, Penv2, also encoded by the *env* gene but at the 3′ end.

Several reports have suggested that differential MMTV *sag* expression affects thymic negative selection and peripheral T-cell activation. For example, the two endogenous MMTV proviruses, *Mtv-1* and *Mtv-6*, encode genetically identical *sag* genes whose products lead to clonal deletion of $V\beta3^+$ T cells; however, only the *Mtv-6 sag* gene results in complete deletion of peripheral $V\beta3^+$ T cells (43). A similar phenomenon was observed for the products of the endogenous *Mtv-7* and *Mtv-43 sag* genes, which are virtually identical, with the same TCR Vβ specificity but with different capacities to stimulate these T cells (51). It is possible that a difference in the levels of expression is responsible for the stimulatory profiles of these two superantigens. *Mtv-7 sag* encodes the strongest endogenous superantigen, and an exogenous homolog, MMTV(SW), with Vβ specificity and superantigen strength identical to those of *Mtv-7 sag* has recently been described (25). Interestingly, however, another novel endogenous provirus, *Mtv-53*, encodes a superantigen that is identical to SW but is much weaker than the *Mtv-7* superantigen (1a). To explain these phenomena, it has been proposed that differences in the levels of *sag* expression are linked to variability of integration sites, i.e., cellular flanking sequences and chromatin structure (1, 57, 60). Differential promoter usage for *sag* expression is an alternative possibility. We propose that the various promoters encoded within MMTV have been conserved to control levels of *sag* expression and tissue specificity; therefore, we should consider promoter mutations an additional variable in explaining functional differences between genetically identical *sag* genes. Our data obtained with the luciferase reporter constructs indicate that the Penv2 promoter is predominantly active in B cells.

Why is this tissue specificity relevant? MMTV superantigen is presented in association with MHC class II on B cells, leading to stimulation of reactive T cells. The resulting release of T-cell cytokines and the production of accessory molecules further stimulate the MMTV-infected B cells. This cellular activation triggers viral replication and results in transport of virus to the mammary tissue (1). Although B-cell *sag* expression appears to be crucial for viral transmission (5), the concentration of superantigen molecules on the surface of this cell is very low (63). An argument to explain this observation is that B cells constitutively express a basal level of superantigen, sufficient to trigger a stimulatory response. Thus, a promoter with minimal activity that perhaps is transcriptionally dependent on factors involved in expression of Ii or MHC class II or other B-cell-specific gene products may play an important function in mediating these crucial events during *sag* expression. The Penv2 promoter has sequence similarities with the promoters of both Ii and MHC class II genes; specifically, they have identical murine S and Y elements with comparable spacing (Fig. 8). The S box in Penv2 probably plays an enhancer role similar to that in the class II and Ii promoters, since we observed only a twofold reduction of promoter activity when the S box was deleted, as has been reported for class II transcription (6).

Absent in Penv2 but present in MHC class II and Ii chain promoters is the X box, an element located between the S and Y boxes that is required for high-level expression of Ii and MHC class II in B cells and also for IFN-γ induction of the same genes in fibroblasts and dendritic cells (10). The binding of transcription factors RFX and NF-Y to the X and Y boxes, respectively, appears to work in a cooperative fashion, leading to functional synergy (49). Interestingly, in Penv2 the cooperative binding complex seems to depend on the IgHep sequence instead of the X box. The B-cell factor that binds to this heptamer is most likely abundant in B cells and may increase in level upon cellular activation.

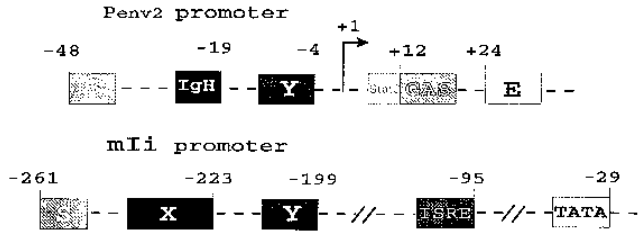

FIG. 8. Comparison of Penv2 and the mouse Ii promoter regions (15). ISRE, interferon-stimulated response element.

Figure 6 suggests that the heptamer (IgHep) sequence is important to Penv2 activity. Heptamer and proximal downstream octamer motifs in the IgH promoter are regulated by a cooperative DNA binding mechanism involving the POU domain-containing proteins Oct-1 and Oct-2, members of a family of gene-regulatory proteins (59). Thus, it is possible that a similar mechanism is operative in the Penv2 promoter, involving the heptamer motif and the Y box. Recent studies of the lipoprotein lipase TATA-less promoter have suggested that binding of Oct-1 and NF-Y to their respective proximal sites is essential for promoter activity; however, no evidence of interaction between these factors has been reported (45). Furthermore, POU-POU interactions may explain the B-cell-specific enhancement of *sag* expression, associated with the downstream U3 region octamer motifs. It is possible that POU proteins binding to the heptamer in Penv2 and to the tandem octamer sites in the U3 region interact, forming a complex which increases *sag* expression. DNA flexibility would be required to allow the proteins that bind to the octamer sites in the U3 region to interact with the protein complex in the promoter region. Our observation that plasmid linearization is necessary to detect the enhancing effect of the downstream octamers supports this model. This enhancement is B cell specific, and the U3 region octamer motifs are in tandem, suggesting that multiple B-cell POU proteins, such as Oct-2 (56) and OCA-B (37), bind to these sites.

The roles of the E box and GAS elements, both located downstream of the start site, are not yet clear. Our results suggest that a deletion of this region reduces promoter activity twofold. This finding supports previous studies of the relevance of promoter elements located downstream of the start site (48); however, it contrasts with data reported on the control of CD2 gene expression, where an upstream E box was shown to be crucial (47). The GAS element in Penv2 is inverted; thus, it remains an open question whether Stat1 or any other related transcription factors can bind to this promoter site, which also encodes an overlapping Stat3 consensus motif between +7 and +13 (Fig. 8). We will explore this possibility in studies of promoter induction by IFN-γ and interleukin 6, which are linked to Stat1 and Stat3 activation (61). The implications for an IFN-γ-inducible promoter linked to the expression of the MMTV superantigen in B cells are of interest with regard to viral transmission.

The data obtained with the luciferase constructs suggest that

Penv2 is active in most cell lines tested with decreasing levels of activity as follows: B cells > fibroblasts > plasmacytoma and JYG mammary tumor cells > T cells. These observations seem consistent with the results obtained in the primer extension experiments, indicating that the *sag* message corresponding to the Penv2 start site is present in most murine cell lines, with the exception of EL-4 T and JYG mammary tumor cells. It is important that only the luciferase reporter activity can be measured in quantitative terms, as the relative concentration of this *sag* mRNA per cell is very low.

Several questions remain to be answered before a role for Penv2 can be formulated. We are in the process of generating silent promoter mutations in the infectious JYG MMTV (67) in order to test their effects on viral transmission. We will also compare differences in levels of *sag* expression by performing functional T-cell proliferation assays with B cells transfected with mutant JYG MMTV clones. These experiments will establish whether Penv2 is used predominantly for basal transcription of *sag*, resulting in T-cell activation. Alternatively, Penv2 may be important for *sag* expression in the thymus as part of the process of negative selection. Evidence suggests that the S, X, and Y boxes are important elements for MHC class II expression in thymic epithelial cells, since CIITA (−/−) mice are defective in CD4 T-cell maturation (10). CIITA has been shown to interact with the transcriptional factors that bind to S, X, and Y boxes, mediating activation of MHC class II expression (50). Since MMTV *sag* and MHC class II genes share the S and Y promoter elements, this indirectly suggests that Penv2 may control the expression of *sag* in the thymus.

The similarities between Penv2 and the Ii promoter lead to the suggestion that MMTV *sag* and Ii share an evolutionary link. In addition to promoter similarities, there are interesting structural resemblances between the MMTV superantigen and Ii: both are type II transmembrane proteins of similar sizes and both associate with MHC class II. It is known that Ii makes use of the CLIP motif to bind to class II (9). We have identified a motif in the *Mtv-7* superantigen that fulfills the requirements for class II binding. The motif lies in a highly conserved region of the extracellular domain of the superantigen about the same distance from the transmembrane segment as the CLIP in Ii. In vitro cotranslation studies suggest a role for this motif in MHC class II binding of the superantigen during biosynthesis (27a). Taken together, these data indicate that the MMTV *sag* gene may have originated from a primordial cellular gene that also gave rise to the Ii gene. It is well established that retroviruses sometimes capture eukaryotic genes during passage in their host and that these genes are maintained only in the viral genome if they provide a survival advantage for the microbe. Since the MMTV superantigen is essential for viral transmission in the mouse, this requirement is fulfilled.

ACKNOWLEDGMENTS

This work was supported by grants from the NIH (R37AI14910) and from the Human Frontiers (RG-544/95M). J. Arroyo was supported by an NRSA (F32) fellowship (AI09197) from the NIAID.

We are grateful to John A. Bottari for technical assistance, Ananda Roy for helpful discussions, Lisa J. Glickstein and Natalie Sutkowski for critical readings of the manuscript, and Robert Underwood for the statistical analyses.

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