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Stimulation of mouse mammary tumor virus superantigen expression by an intragenic enhancer

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ABSTRACT **The mechanisms regulating expression of mouse mammary tumor virus (MMTV)-encoded superantigens from the viral *sag* gene are largely unknown, due to problems with detection and quantification of these low-abundance proteins. To study the expression and regulation of the MMTV *sag* gene, we have developed a sensitive and quantitative reporter gene assay based on a recombinant superantigen–human placental alkaline phosphatase fusion protein. High *sag*–reporter expression in Ba/F3, an early B-lymphoid cell line, depends on enhancers in either of the viral long terminal repeats (LTRs) and is largely independent of promoters in the 5′ LTR. The same enhancer region is also required for general expression of MMTV genes from the 5′ LTR. The enhancer was mapped to a 548-bp fragment of the MMTV LTR lying within *sag* and shown to be sufficient to stimulate expression from a heterologous simian virus 40 promoter. No enhancer activity of the MMTV LTR was observed in XC sarcoma cells, which are permissive for MMTV. Our results demonstrate a major role for this enhancer in MMTV gene expression in early B-lymphoid cells.**

Mouse mammary tumor virus (MMTV) is a murine B-type retrovirus that causes a high incidence of mammary tumors in infected females. Infectious MMTV is transmitted from mother to offspring via milk (see refs. 1 and 2 for review). Infection of B lymphocytes plays an important role in the viral life cycle (3, 4). Also, endogenous MMTV mRNAs are found in pro-B, pre-B, and mature stages of B-cell development (5).

MMTV encodes a superantigen (Sag protein) that, when expressed on the surface of B cells or other antigen-presenting cells, activates a large number of T cells by interaction with specific T-cell receptor β chains (6). The resulting T-cell response in turn stimulates the infected B cells to proliferate (4) and thus amplifies the number of virus-infected cells. The viral *sag* gene encoding Sag is located within the viral long terminal repeat (LTR). All current evidence indicates the use of the 3′ LTR *sag* gene in superantigen expression. Three different promoters have been implicated in *sag* expression under different conditions: the classical promoter at the U3–R border (P_1) (7, 8), which is used for the expression of all retroviral genomes and structural genes (9); a promoter within U3 (P_2) (10); and a phorbol ester-inducible promoter within the *env* gene (P_{env}) (11, 12). The results of these experiments have to be interpreted with caution because (*i*) mRNAs characterized by reverse transcription–PCR may represent rare aberrant transcripts irrelevant for the total level of *sag* expression, (*ii*) not all RNAs containing the *sag* gene are necessarily used for Sag protein expression, and (*iii*) promoter activity of isolated subgenomic regions might not be important in the context of a complete provirus.

The lack of a sensitive and quantitative assay for superantigens has prevented a detailed understanding of *sag* gene regulation. Detection of superantigen expression has been achieved with either a functional test for superantigen activity or monoclonal antibodies. Both techniques are of only limited use. Superantigen activity depends on coexpression of major histocompatibility complex (MHC) class II molecules on the same cell, and MHC class II molecules are known to limit the functional expression of MMTV superantigens (13). Detection of Sag proteins by monoclonal antibodies is relatively insensitive (14).

To determine the viral sequences regulating *sag* gene expression in a MMTV provirus and to quantify their effects, we developed a sensitive *sag* gene reporter assay. We found that high expression of the *sag* gene in an early B-lymphoid (pro-B) cell line is largely independent of the viral 5′ LTR and relies on an intragenic enhancer element.

MATERIALS AND METHODS

Plasmids. The *Mtv-1*/C3H recombinant proviral clone "hybrid MMTV" in pBR322 (15) was a gift from H. Varmus (National Institutes of Health). References to nucleotide positions are based on *Mtv-1* LTR (16) and C3H LTR (17) for 5′ and 3′ LTRs and on MMTV(BR6) (18) for the internal region. Plasmid pBC12/PLAP 513 (19), containing a cloned human placental alkaline phosphatase (PLAP) cDNA, was kindly provided by S. Udenfriend (Roche Institute of Molecular Biology). The β-galactosidase expression plasmid pCMVβ (20) was obtained from B. Huber (Tufts University).

Recombinant Viral Constructs. The hybrid MMTV provirus was transferred into pGEM-2 (Promega) by using the *Eco*RI and *Hin*dIII sites in the flanking DNA (plasmid pM). Recombinant PLAP genes with and without the initiation codon were generated by PCR using *Pfu* DNA polymerase (Stratagene) under standard conditions with primer FP5 (5′-GACTAGTC-CAAGCTTCTGCATGCT-3′) or FP5.2 (5′-GACTAGTCT-GCTGCTGCTGCTGCTGGGC-3′), primer FP3 (5′-AGCCCCCTTAAGCGGCCGCTCAGGGAGCA-3′), and template pBC12/PLAP 513. The products were either cut with *Spe* I and *Afl* II and ligated to the *Avr* II and *Afl* II sites in the 3′ LTR of the hybrid MMTV provirus (pM1^{sapF}, pM1^{sap}) or cut with *Hin*dIII and *Afl* II and inserted after ligation to *Avr* II/*Hin*dIII adaptors (pM1^{sapS}). Plasmid pM^{sap} contains the (C3H) LTR *Avr* II–*Afl* II fragment cloned into *Not* I and *Afl* II sites of pM1^{sap}. PLAP genes were isolated as a *Hin*dIII–*Not* I fragment from pM1^{sapF} (wild-type PLAP) or *Bgl* II–*Not* I fragments (*sap, sapF, sapS*) from the respective proviral construct and cloned into the *Hin*dIII and *Not* I sites of pRC/CMV (Invitrogen) by using *Bgl* II/*Hin*dIII adaptors where necessary. Proviral 5′ truncations were done by using either the *Eco*RI site in pGEM-2 and the *Stu* I (nt 556), *Sty* I (nt 780), *Rsa* I (nt 828), *Sty* I (nt 963), *Eco*RI (nt 5803), *Cla* I (nt 7478), and *Bgl* II (nt 8532) sites in MMTV or the *Pst* I site in pGEM-2 and *Pst* I sites (nt 9 or 1459) in MMTV. pM2^{sap} constructs were derived from pM1^{sap} by deletion of nt 1464–8532. PCR-ampli-

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Abbreviations: LTR, long terminal repeat; MHC, major histocompatibility complex; MMTV, mouse mammary tumor virus; PLAP, placental alkaline phosphatase.

fied *Mtv-1* LTR fragment 9–556 with flanking *Not* I sites was cloned into either the *Not* I site of the 3′ LTR or the *Eco*RI site of the 5′ LTR of pM1^{sap} Δ554 or into the *Xho* I or the *Bam*HI site in pGL2-promoter (Promega).

Cell Lines and Transfection. The rat sarcoma cell line XC was obtained from N. Hopkins (Massachusetts Institute of Technology) and cultured as described (15). The interleukin 3-dependent mouse pro-B cell line Ba/F3 (21), a gift from U. Klingmüller (Whitehead Institute), was maintained as described (22). DNA was introduced into Ba/F3 cells by electroporation with a Bio-Rad Gene Pulser and into XC cells by lipofection (Lipofectin; GIBCO/BRL). Equimolar amounts of test plasmids (1 pmol for XC, 2 pmol for Ba/F3 cells) were linearized outside of the cloned provirus. After addition of 0.1 pmol (XC) or 1–3 pmol (Ba/F3) of control plasmid pCMVβ, carrier plasmid pGEM-2 and TE (10 mM Tris/1 mM EDTA, pH 7.0) were added to give a constant DNA amount and volume.

Reporter Gene Assays. Cells were washed twice in isotonic salt solution (154 mM NaCl/50 mM Tris·HCl, pH 7.5/1 mM $MgCl_2$) and resuspended in 300 μl of the same buffer. A sample (50–100 μl) was removed and processed for β-galactosidase assay, and the remainder was incubated for 30 min at 65°C. For the alkaline phosphatase assay (triplicate determinations), 8 μl of cell suspension was added to 100 μl of reaction mixture [0.95 M diethanolamine/0.28 M NaCl/0.5 mM $MgCl_2$/0.4 mM CSPD (Tropix, Bedford, MA)/0.1% Sapphire (Tropix)/10 mM EDTA, pH 9.85] and incubated at room temperature for 20 min. Light emission was determined for 10 sec in an ILA911 luminometer (Tropix). β-Galactosidase levels were quantified in triplicate assays using the Galacto-light system (Tropix). The luciferase assay system (Promega) was used for detection of firefly luciferase (duplicate assays).

RESULTS

Sag–PLAP Fusion Protein as Superantigen Reporter. Quantitative assay of the MMTV *sag* gene product expressed from a provirus has not been possible with available methods (13, 14). To study the regulation of *sag* gene expression in MMTV, we inserted a reporter gene encoding human PLAP into the *sag* gene of an infectious and oncogenic MMTV provirus (15), thus creating a hybrid *sag*–PLAP gene (*sap*) in the 3′ LTR (Fig. 1). Since expression of functional Sag requires translational initiation at the codon for Met-1 or Met-38 within the *sag* gene (23), with initiation at Met-1 being greatly preferred over Met-38 in *in vitro* translation studies (24), a PLAP gene lacking its original initiation codon was inserted in-frame after Sag codon 17 (pM^{sap}). All MMTV proviral sequences are present in this construct. Expression of the reporter gene in this system is expected to result in the translation of a recombinant alkaline phosphatase with the N-terminal 17 aa of the C3H Sag protein at its N terminus.

To determine whether the product of the *sag*–PLAP reporter gene retains its enzymatic activity, we cloned the recombinant *sap* gene and 5′ flanking viral leader sequence into the expression plasmid pRC/CMV (pRC/CMV*sap*). After transfection of this plasmid into XC cells (Fig. 2), PLAP activity was 4- to 5-fold higher than in cells transfected with the positive control pRC/CMV.PLAP, which retained the original

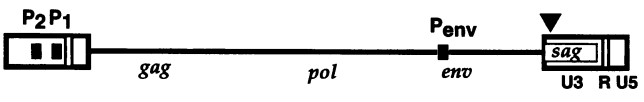

FIG. 1. Schematic representation of the MMTV provirus. The position of viral genes (*gag, pol, env*, and *sag*), promoters (P_1, P_2, and P_{env}), and functional regions within the LTRs (U3, R, U5) are indicated. An arrowhead denotes the position of the human PLAP reporter gene in the 3′ LTR.

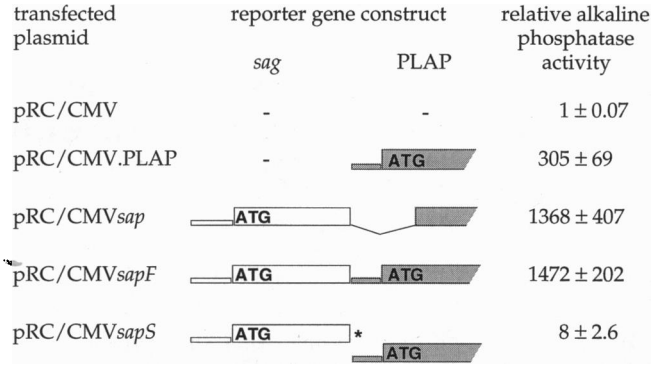

FIG. 2. Alkaline phosphatase activity in XC cells 48 hr after transfection with pRC/CMV plasmids encoding wild-type PLAP or hybrid Sag–PLAP. Alkaline phosphatase activities have been corrected for β-galactosidase activity expressed from cotransfected pCMVβ and have been normalized to pRC/CMV activity = 1. Numbers represent the arithmetic mean ± SD of three separate experiments with two different DNA preparations. The N-terminal portions of the *sag* (open boxes) and PLAP genes (shaded boxes) are shown with translational initiation sites (ATG), stop codons (*), and 5′ flanking sequences (small boxes). The gap in pRC/CMV*sap* indicates the lack of the PLAP Met-1 initiation codon.

PLAP leader sequence and initiation codon. The presence of the PLAP initiation codon and leader sequence in addition to, and in frame with, the *sag* initiation codon did not alter PLAP activity (pRC/CMV*sapF*). In the same context, termination of the recombinant protein after the Sag portion by introduction of a stop codon and frameshift mutation greatly reduced PLAP activity (pRC/CMV*sapS*). We conclude that the hybrid reporter gene *sap* encodes an enzymatically active alkaline phosphatase. The reporter activity depends on an intact *sag*–PLAP reading frame. These findings strongly suggest that expression of the *sag*–PLAP reporter within the MMTV provirus M^{sap} is regulated in the same way as *sag* expression.

***sag*–Reporter Activity Is Largely Independent of 5′ LTR and Is Differentially Regulated in Ba/F3 and XC Cells.** The question of cell type-specific regulation of superantigen expression was addressed by transient transfection of the proviral *sag*–reporter construct pM^{sap} into the rat sarcoma cell line XC and the mouse pro-B cell line Ba/F3. XC cells are permissive for MMTV infection and commonly used for infection and expression studies (15). The bone marrow-derived pro-B cell line Ba/F3 (21) has been chosen as an early B-cell model system because high transfection efficiency and low cellular alkaline phosphatase levels allow sensitive *sag*–reporter detection. PLAP levels after pM^{sap} transfection were ≈450-fold (Ba/F3) and ≈5-fold (XC) increased over cellular background levels (Fig. 3).

To determine the location within the provirus of regulatory elements required for *sag* gene expression, we created a series of deletion constructs (pM^{sap} Δ) lacking increasing portions at the 5′ end of the provirus. In Ba/F3 cells (Fig. 3*A*) partial or complete removal of the 5′ LTR did not result in a drastic change in *sag*–reporter activity. Deletion mutants pM^{sap} Δ9, Δ556, Δ780, and Δ828 retained reporter activities equivalent to or slightly higher than the intact pM^{sap}. Constructs with extended truncations—pM^{sap} Δ963, Δ1456 (lacking the entire 5′ LTR), and Δ5803—consistently showed moderately reduced values of 70–80% of the initial activity. By contrast, further deletion of nt 5803–7478, removing the putative *env* promoter, sharply reduced the reporter signal to 9% of wild type. The signal from plasmid pM^{sap} Δ8532, with only 41 nt of viral sequence left upstream of the Sag initiation codon at nt 8573, remained at this level. Further truncation of the provirus to nt 8567 did not decrease the signal, and removal of vector sequences 5′ of the provirus by linearization of the plasmid at

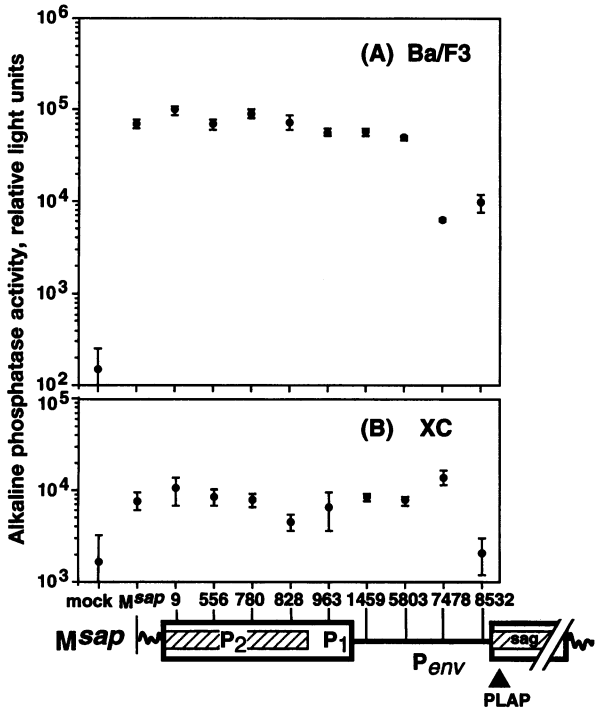

FIG. 3. Mapping of regions in MMTV that are involved in superantigen expression. Ba/F3 (*A*) or XC (*B*) cells were transfected with plasmid pM^{sap} or one of the truncated versions pM^{sap} Δ9, Δ556, Δ780, Δ826, Δ963, Δ1459, Δ5803, Δ7478, and Δ8532. Alkaline phosphatase activities have been corrected for β-galactosidase activity of cotransfected pCMVβ and expressed as the arithmetic mean ± SD of at least three separate experiments with two different DNA preparations. The viral constructs used are depicted below the graphs. Numbers identify the deleted 5′-terminal portions of the provirus. Positions of previously described promoters (P_1, P_2, P_{env}), the *sag* gene (hatched box), the PLAP gene, and nonviral flanking DNA (wavy line) are indicated.

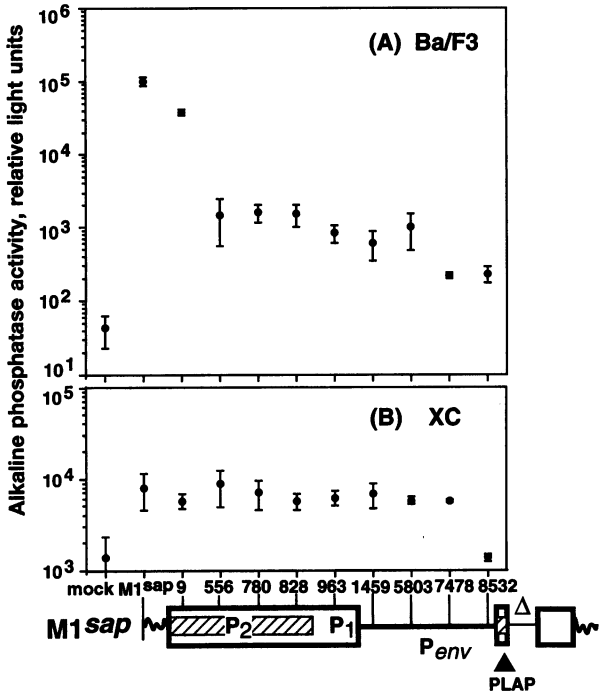

FIG. 4. Effect of the 3′ LTR on regulation of MMTV superantigen expression. Ba/F3 (*A*) or XC (*B*) cells were transfected with plasmid pM1^{sap} or one of the truncated versions pM1^{sap} Δ9, Δ556, Δ780, Δ826, Δ963, Δ1459, Δ5803, Δ7478, and Δ8532. The deletion of nt 54–991 in the 3′ LTR (Δ) is indicated. For data calculation and designations see Fig. 3.

nt 8552 or 8567 had only a weak (2-fold) effect (data not shown), excluding the presence of strong promoters in these regions upstream of the *sag* gene.

The *sag*–reporter activity of pM^{sap} in XC cells (Fig. 3*B*) was not significantly affected by the deletion of the 5′ LTR and internal genes up to nt 7478. Removal of nt 7478–8532 reduced the reporter levels by a factor of 4–5 down to mock control levels, suggesting the presence of an as yet uncharacterized weak promoter within this region. In summary, *sag* gene expression in both cell lines in the absence of added steroid hormones is largely independent of the 5′ LTR and relies in both cases on regions within the *env* gene for expression. Significant *sag*–reporter expression in Ba/F3 cells after removal of the 5′ LTR and most internal sequences suggested the presence of additional stimulatory signals within the viral 3′ LTR. Such residual activity was not noticed in XC cells.

High *sag*–Reporter Activity in Ba/F3 Cells Depends on a Cell Type-Specific Regulatory Element Within Either LTR. To test the possible contribution of regulatory elements within the 3′ LTR to *sag* gene expression, we generated a proviral *sag*–reporter construct lacking nt 54–991 of the 3′ LTR (pM1^{sap}). Signals for polyadenylylation of MMTV transcripts were not affected. pM1^{sap} reporter levels after transfection of both Ba/F3 and XC cells were comparable to reporter levels seen with the complete pM^{sap} construct (Fig. 4).

In Ba/F3 cells (Fig. 4*A*) truncation of the pM1^{sap} provirus from the 5′ end revealed a striking difference from the constructs with intact 3′ LTRs. The *sag*–reporter expression decreased modestly (factor of 2.5) after removal of nt 1–9 of the 5′ LTR and flanking region (pM1^{sap} Δ9). In contrast, a sharp reduction by a factor of 25, to 1.5% of wild-type activity, occurred when nt 9–556 of the 5′ LTR were deleted (pM^{sap} Δ556). Further truncations removing the putative P_2 promoter (pM1^{sap} Δ780 and Δ828) had no effect (1.6% and 1.5% of initial activity). Further deletion including the entire 5′ LTR or entire 3′ half of the provirus decreased the signal to ≤1% of the initial value, most likely due to inactivation of the classical promoter. Reporter activity in deletion constructs pM1^{sap} Δ7478 and Δ8532, both lacking the putative promoter P_{env}, was further reduced to 0.2% of wild-type levels, close to the mock control. These results indicate that the 5′ LTR from nt 9 to nt 556 contains a regulatory element that is required for high level *sag* expression in Ba/F3 cells. As shown before (Fig. 3*A*) 5′ LTR nt 9–556 could be functionally replaced by nt 54–991 in the 3′ LTR. The localization of a stimulatory element to overlapping regions in the 5′ and 3′ LTRs suggests that the effect was mediated by the same element acting from different positions.

The results for pM1^{sap} deletion constructs in XC cells (Fig. 4*B*) were identical to those obtained with the pM^{sap} constructs, indicating no effect of 3′ LTR nt 54–991 on *sag* expression in these cells. Therefore, *sag* gene expression is differentially regulated in Ba/F3 and XC cells.

Viral Gene Expression from the 5′ LTR in Ba/F3 Cells Depends on a Cell Type-Specific Regulatory Element in the LTR. The requirement of LTR fragment 9–556 for high *sag* expression in Ba/F3 cells raises the question whether the same regulatory element is required for expression of other viral products in this cell line. To test expression of viral genes that are controlled by a promoter within the 5′ LTR, such as *gag*, *pol*, and *env*, we generated the reporter construct pM2^{sap}. The reporter gene in pM2^{sap} was placed 175 nt downstream of the 5′ LTR by deleting most of the internal proviral region from pM1^{sap}. The results from pM2^{sap} (Fig. 5) show that the alkaline phosphatase signal resulting from an intact 5′ LTR in Ba/F3 cells was reduced by a factor of 100 after removal of nt 1–556 in the 5′ LTR and 5′ flanking sequences. The flanking region

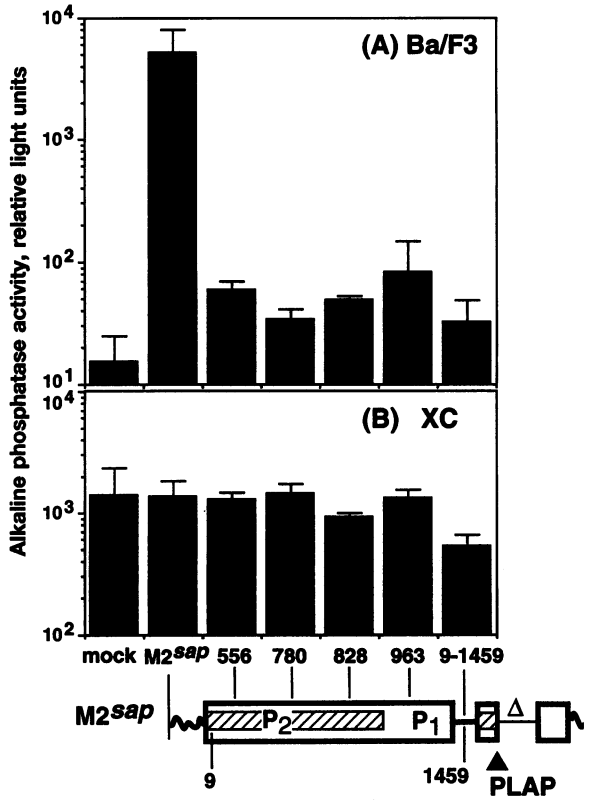

FIG. 5. Identification of regions involved in gene expression from the 5′ LTR of MMTV. Ba/F3 (*A*) or XC (*B*) cells were transfected with plasmid pM2^{sap} or one of the truncated versions pM2^{sap} Δ556, Δ780, Δ826, Δ963, and Δ9–1459. The deletion of nt 54–991 in the 3′ LTR (Δ) is indicated. For data calculation and designations see Fig. 3.

was not sufficient to confer detectable reporter expression. No pM2^{sap} reporter activity was detectable in XC cells. We conclude that LTR fragment 9–556 is also required for expression of MMTV genes other than *sag* in Ba/F3. The overall lower reporter levels of pM2^{sap} compared with pM1^{sap}, including the internal proviral region (Fig. 4), is consistent with the presence of a promoter in the internal region.

The MMTV LTR Contains an Enhancer Active in Ba/F3 Cells. The position-independent stimulatory effect of 5′ LTR nt 9–556 or 3′ LTR nt 54–991 on *sag* gene expression in Ba/F3 cells was suggestive of the presence of an enhancer element within these regions. To test this hypothesis, we used plasmid pM1^{sap} Δ556, which lacks these stimulatory regions and exhibits a low level of *sag*–reporter activity in Ba/F3 cells (Fig. 6). We reintroduced the *Mtv-1* LTR fragment 9–556 into either the 5′ or the 3′ LTR of pM1^{sap} Δ556. When located in the 5′ LTR, the fragment in both orientations stimulated *sag*–reporter expression 20-fold, to levels comparable to pM1^{sap}. When the fragment was present in the 3′ LTR the stimulation was stronger in the minus orientation (25-fold) than in the plus (original) orientation (8-fold). These experiments confirm that *Mtv-1* LTR fragment 9–556 acts as position- and orientation-independent enhancer element to stimulate *sag*–reporter activity in Ba/F3 cells.

***Mtv-1* LTR Fragment 9–556 Is Sufficient as Enhancer in Ba/F3 Cells.** To determine whether *Mtv-1* LTR fragment 9–556 contains all the necessary elements required for enhancer activity or depends on other elements within the provirus, we tested the enhancer activity of this region in a heterologous system. In pGL2-promoter the firefly luciferase gene is under the control of the simian virus 40 promoter and, due to the lack of enhancers, only weakly expressed. The low basal level of luciferase expression from this plasmid was increased 30-fold by introduction of *Mtv-1* LTR fragment

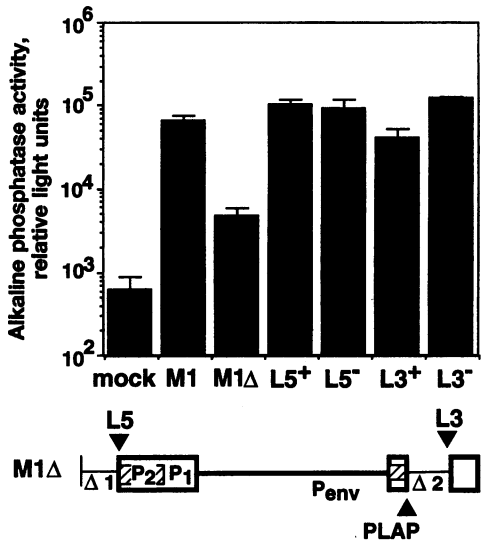

FIG. 6. LTR fragment 9–556 is active as an enhancer in the MMTV provirus. Ba/F3 cells were transfected with pM1^{sap} (M1), pM1^{sap} Δ556 (M1Δ) or pM1^{sap} Δ556 containing *Mtv-1* LTR nt 9–556 in either the 5′ LTR (L5) or the 3′ LTR (L3); + and − denote the orientation of the LTR fragment. Alkaline phosphatase activities were determined as described in the legend to Fig. 3. The viral construct pM1^{sap} Δ556 (M1Δ) is depicted below the graph. Deletions of nt 1–556 from the 5′ LTR (Δ1) and nt 56–1035 from the 3′ LTR (Δ2), positions of inserted fragments (L5, L3), previously described promoters (P_1, P_2, P_{env}), the *sag* gene (hatched box), and the PLAP gene are indicated.

9–556 into the position 3′ from the luciferase gene, independent of the fragment orientation (Fig. 7). The effect was increased up to 2-fold when the LTR fragment was placed upstream of the luciferase gene, indicating weak position or promoter effects. These results provide strong evidence for the presence of a complete enhancer element in *Mtv-1* LTR fragment 9–556, capable of at least 30-fold stimulation of expression in Ba/F3 cells.

DISCUSSION

No sensitive assay has been available to detect and quantitate expression of the MMTV *sag* gene independent of MHC class

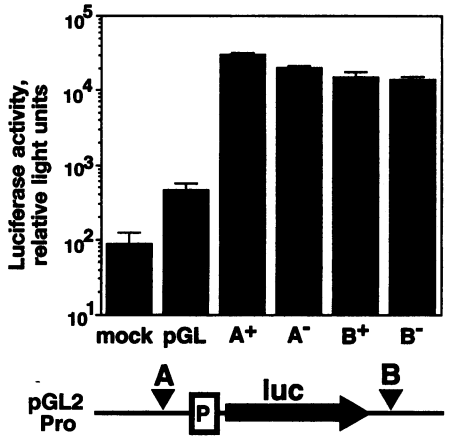

FIG. 7. LTR fragment 9–556 contains a complete enhancer element. Ba/F3 cells were transfected with pGL2-promoter (pGL2-Pro) or pGL2-Pro carrying the Mtv-1 LTR fragment 9–556 in a position 5′ (A) or 3′ (B) from the luciferase (luc) gene; + and − denote the orientation of the LTR fragment. The relative luciferase activity is expressed as the arithmetic mean ± SD of four transfections with two different DNA preparations. pGL2-Pro is depicted below the graph. Positions of inserted fragments (A, B) and simian virus 40 promoters (P) are indicated.

II expression. We have established a sensitive, quantitative, and MHC class II-independent reporter assay for the expression of the MMTV *sag* gene that is based on the enzymatic activity of a Sag–PLAP fusion protein. Increased sensitivity through the use of a chemiluminescent substrate and luminometric analysis enabled us to detect and quantitate PLAP-linked proteins over a very wide dynamic range. Since the presence of the intact *sag* gene is not required for this assay, regulatory regions within *sag* itself could be tested for their effect on *sag* expression.

Relatively little is known about regulation of MMTV transcription in cells of the B-cell lineage. Endogenous MMTV mRNAs are found in normal cells or cell lines representing pro-B, pre-B, and mature stages of B-cell development (5), and their expression can be induced in mature B cells (25). However, the putative enhancers, transcription factors, and binding sites involved are still elusive.

In this study we tested the regulation of MMTV *sag* gene expression in two selected cell lines in the absence of added steroid hormones and detected a MMTV enhancer activity in B-lymphoid cells. In Ba/F3, a bone marrow-derived early B-lymphoid cell line without known superantigen activity, cellular factors are present that interact with LTR nt 9–556 to enhance expression of superantigens and other viral proteins at least 20-fold. The enhancer is not active in rat XC cells, which are permissive for MMTV infection and expression (15), presumably due to the absence in these cells of one or more cell type-specific factors. The enhancer element localizes to the 5′ end of the MMTV LTR, overlapping with codons 4–183 of the *sag* gene, and thus represents an intragenic enhancer. Further experiments are required to determine whether this enhancer shares transcription factor-binding sites with a mammary gland cell-specific enhancer previously mapped within this general region (26, 27). The MMTV enhancer activity in B-lymphoid cells ensures viral gene expression in cells that do not tolerate the high concentrations of steroid hormones necessary for high MMTV expression in other tissues.

Almost all retroviral genes are transcribed from a promoter within the 5′ LTR. The primary transcript is either directly used for translation of *gag* and *pol* genes or spliced to allow expression of *env* and possible accessory genes. In MMTV the situation may be different. In a MMTV provirus lacking the entire 5′ LTR, we have found that a region within the *env* gene is sufficient for *sag* expression equivalent to 70–80% of wild-type activity in the presence of the 3′ LTR. Similar observations have been made for *Mtv-7* superantigen activity in a B-cell hybridoma (28). Promoters within the 5′ LTR are apparently not required for *sag* gene expression. The position of the *env* gene region critical for reporter activity suggests the involvement of the previously described *env* gene promoter (P_{env}) (11, 12). A contrasting recent report demonstrates that the endogenous provirus *Mtv-6* expresses sufficient Sag protein to induce superantigen-dependent T-cell deletion (8) but lacks most of the *env* gene, including P_{env}. Interestingly, our mutant provirus $M2^{sag}$ (Fig. 5), which has a similar, even larger deletion of the internal viral region, exhibits a reduced but still significant *sag*–reporter signal that may be sufficient *in vivo* to induce the observed superantigen effects. These results are consistent with a concerted action of the classical LTR promoter P_1 and P_{env} for *sag* expression in an intact provirus. Inactivation or loss of one promoter could be compensated for by the second promoter. Dual expression from the classical promoter in the 5′ LTR or a promoter within the *env* gene has previously been demonstrated for the *bel-1/taf* genes of spumaviruses (29, 30).

The presence of newly integrated proviruses in the bone marrow of mice infected with exogenous MMTV (31) strongly suggests a functional relevance of MMTV gene expression in bone marrow-derived cells such as Ba/F3. It remains to be determined whether *sag* expression in mature B cells is regulated in the same way. Our *sag*–reporter assay provides the experimental tool to address this question.

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