Regulated Expression of Mouse Mammary Tumor Proviral Genes in Cells of the B Lineage

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Summary

We evaluated the expression of mouse mammary tumor proviral (MMTV) transcripts during B cell ontogeny and compared levels of RNA in B lymphocytes and B cell lines with levels in other cells of the hematopoietic lineage and in a mammary cell line. We demonstrate that MMTV transcripts are expressed as early as the pro-B cell stage in ontogeny and are expressed at basal constitutive levels throughout most of the B cell developmental pathway. The level of MMTV expression in B cells is similar to constitutive levels in mammary tissues and two to three orders of magnitude greater than in activated T cells. Levels of MMTV transcripts in B cells are not solely due to positional effects. Transient transfection assays showed that MMTV upregulation resulted from transcriptional activation of the viral LTR, indicating that there are specific and inducible transcription factors that regulate MMTV expression in B cells. MMTV transcripts could not be upregulated in pre-B cell lines but could be induced in some mature B cell lines. There was a correlation between the ability to stimulate B cells to secrete antibody and the ability to induce upregulated MMTV expression. Evidence is presented that suggests that the principal transcription factors involved in MMTV expression do not include the B cell factors OTF-2 or NF-κB, but rather are likely to be novel factors that are induced during differentiation to antibody secretion. A hypothesis for why mammary tumor viruses are well adapted for expression in cells of the B lineage is proposed, and the implications of this for the documented influence of MMTV gene products on the T cell repertoire are discussed.

Mouse mammary tumor virus (MMTV) is a type B morphology retrovirus that can be transmitted either exogenously as a retroviral particle or endogenously via the germ line as an integrated provirus (1). More than 20 MMTV proviruses have been described, 3-6 of which are found in any given inbred strain of mice (2, 3). Two of these, Mtv-1 and Mtv-2, are associated with mammary adenocarcinomas (1, 3). The transcriptional regulation of MMTV in mammary epithelial cells has been extensively studied, and has been found to be absolutely dependent on the binding of hormones to their respective receptors, which allows receptor translocation to the nucleus (4). The binding of these receptors to defined sequences within the glucocorticoid response element (GRE) of the U3 region of the viral long terminal repeat (LTR) (5) alters the chromatin structure, allowing initiation of transcription (6). Other transcription factors, including NF-1 (7, 8) and OTF-1 (9), can then bind to their respective sites and activate high levels of MMTV transcription. The GRE also contains the sequences that contribute to the tissue specificity of proviral expression (reviewed in reference 10). While several laboratories have shown that constitutive, high level expression of MMTV transcripts can be found in various tumor cell lines that are not hormonally stimulated (11-15), all of these cells show evidence of proviral amplification and/or the expressed transcripts contain deletions or substitutions within the U3 region of the LTR. The region altered in the amplified proviruses is thought to play a role in conferring tissue specificity by negative regulation (14).

We (16-18) and others (19, 20) have demonstrated that MMTV can be expressed in at least some B cell lines and in activated normal B cells. We have identified three novel features which distinguish the mechanisms regulating MMTV expression in B cells from those regulating its expression in non–mammary tumor cells and in mammary epithelium. First, the expression of MMTV transcripts in B cells does not result from proviral amplification or from alterations within the LTR (16, 17). Sequence analysis revealed that the U3 region of the LTR in expressed transcripts contains neither deletions nor insertions, and the known regulatory sites, including the GRE, are not disrupted (17). Second, both constitutive levels of MMTV transcripts and the increased levels induced upon LPS stimulation are not dependent on
binding of glucocorticoid receptor (GR) to sequences in the GRE (18). Third, MMTV expression is not limited to the proviral loci that are known to be expressed in mammary tissue, including Mtv-1 and Mtv-2 (16, 17). Together, these data suggest that B cells express normal cellular factors which regulate MMTV expression in a novel way, independent of GR-GRE interactions.

Several laboratories have recently demonstrated a close genetic linkage between a number of endogenous MMTV loci and the ability of a given mouse strain to express superantigens or cotoligenes that are responsible for the activation or deletion of T cells expressing receptors with a particular Vβ phenotype (21–24). We have directly demonstrated that at least three of the endogenous proviral loci (Mtv-6, Mtv-8, and Mtv-9) encode superantigens (25). There is also direct evidence that viral DNA from the infectious forms of MMTV encode such deleting elements (26–28). In light of these observations, it is clearly important to gain a more thorough understanding of the regulation of MMTV expression in B cells, and to determine if other cells of the hematopoietic lineage also are capable of expressing MMTV gene products.

In this report, we show that MMTV transcripts are primarily, but not exclusively, detected in cells of the B lineage, and that constitutive low levels of MMTV transcripts are expressed from the earliest stage of B cell ontogeny examined, the pre-B cell stage. Our data also demonstrate that the ability to stimulate increased levels of MMTV transcripts in B cells is due to transcriptional activation controlled by sequences within the proviral LTR. Finally, we demonstrate that the upregulated expression of MMTV above basal constitutive levels is only observed in mature B cells that are capable of differentiating into antibody-secreting cells. The implication of these results for the expression of MMTV in cells of the B lineage, and the effects of MMTV gene products on the T cell repertoire, are discussed.

Materials and Methods

Cell Lines and Tissue Culture Conditions. All cell lines were cultured in supplemented DMEM prepared using endotoxin-free water and containing 10% FCS (J. Scientific, Woodland, CA) (16, 17). The CH12-LBK cell line, an in vitro adapted subclone of the CH12 B cell lymphoma, has been previously described (29). All B cells and B cell lines were stimulated for varying periods of time with LPS (Escherichia coli 055:B5), Difco Laboratories Inc., Detroit, MI) or lymphokines (IL-2, 50 U/ml, or IL-5, 25 U/ml) as described (17). Lymphokines were purchased from Genzyme Corp. (Cambridge, MA).

A number of cell lines representing various developmental stages in the hematopoietic lineage were used, and some of these have been previously reported (17). Cell lines new to this study and their reported stages of development are: NFS-70, a pro-B cell; NFS-25, an early pre-B cell line; 15-79-2, pre-B cell, and ABE-8.1/2 and 2E8, both considered late pre-B cells; BCL-1, WEHI-231, and A20, all surface Ig - B cells; R1.1 and BW5147, both T cell lymphomas; WEHI-3, considered to be an immature stem cell of myelomonocytic character; RAW-309Cr.1, a monocyte-macrophage cell line; and IC-21 and RAW-264, both macrophage cell lines. All cell lines were obtained from the American Type Culture Collection (Rockville, MD), except 2E8, which was provided by P. Kincade (30; Oklahoma Medical Research Foundation, Oklahoma City, OK), and A20 and one of the WEHI-231 sublines, which were provided by M.P. Hap and E. Palmer (National Jewish Center for Immunology, Denver, CO). The mammary epithelial cell line MMT60562 (MMT) was grown without 2-ME.

Isolation of Normal B and T Cells. Mice were used at 6–12 wk of age. B10.A mice were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). All other strains, C57BL/6, BALB/c, CBA/J, and DBA/2, were from The Jackson Laboratory (Bar Harbor, ME). Enriched populations of splenic B cells were prepared as described (18) and stimulated at 2 x 10^6 cells/ml for either 24 or 84 h. T cells were prepared from B10.A spleen cells as follows: biotin-labeled anti-Ig5/B220 antibody (PharMingen, San Diego, CA) was mixed with Dynabead strepavidin conjugates (DYNAL Inc., Great Neck, NY) and incubated with spleen cells (depleted of macrophages by adherence) at a ratio of two beads to one cell for 30 min. Cells bound by the beads were removed using a magnet. Nonadherent cells were retreated a second time with the anti-B220 and magnetic beads. The enriched T cells (5 x 10^6/ml) were stimulated with 5 μg/ml Con A (Calbiochem Corp., La Jolla, CA); 10 U/ml IL-2 was added after 18 h. After 72 h, the cells were retreated with anti-B220-coupled magnetic beads to remove residual B cells. These T cell preparations were determined to be essentially free of contaminating B cells by the failure to detect Ig κ sequences on Northern blot analysis of T cell RNA.

RNA Preparation and Northern Blot Analysis. Total cellular RNA was extracted from cell lines or normal lymphocytes as described (17). RNA was isolated from tissues by the method of Chirgwin et al. (31). Northern blot analysis was performed after electrophoresis of RNA in 1% formaldehyde-agarose gels and transfer of RNA to Nitroplus 2000 membranes (MSI, Westborough, MA) for probing as described (17).

Isolation of Genomic DNA and Southern Blot Analysis. High molecular weight genomic DNA was isolated and Southern blot analysis was performed using ammonium acetate for transfer of DNA to Hybond nylon membranes (Amersham Corp., Arlington Heights, IL) as described (17). Methylation experiments were performed as described in Results. Control digests using the methylation insensitive enzymeMspI were performed to confirm that all DNA samples gave rise to the same restriction pattern. All restriction enzymes were purchased from Boehringer Mannheim Corp. (Indianapolis, IN).

Probes. All cDNA probes were isolated as inserts and labeled by random priming (18) according to the Oligolabeling kit specifications (Pharmacia LKB Biotechnology Inc., Piscataway, NJ). The following probes were used: MMTV LTR, pA7; MMTV envelope (env), pSC34; MMTV (env + LTR), pA8; Ig κ, μ, μ107; actin, pHF1. All of these have been described (16–18, 29). Also used were an Ig κ probe, a 500-bp EcoRI insert from pSC33, encoding the 3' portion of the κ constant region; OTF-2, a 1.9-kb XbaI/BamHI insert from the pOEV + construct (32; provided by L. Eckhardt, Columbia University, New York), and CHO-B, a 600-bp EcoRI/BamHI fragment from the CHO-B plasmid (33; provided by R. Wall, UCLA). Northern blots were washed with 2x SSC/0.1% SDS at 65°C. Southern blots were washed at 67°C in 2x SSC/0.1% SDS followed by washes at 68°C in 0.1x SSC/0.1% SDS.

DNA Constructs for Transformation. A Mtv-9 luciferase reporter construct was prepared as follows: A 1,200-bp PstI/EcoRI fragment containing the entire Mtv-9 LTR was isolated from clone pA8 (17). The insert was subcloned into pGEM 4Z (Promega Corp., Madison,
WI) and then excised with HindIII and EcoRI. The EcoRI end was modified with an EcoRI/BamHI linker (provided by M. Ostrowski, Duke Medical Center, Durham, NC) and the LTR was subcloned directionally into the HindIII-BglII sites of the luciferase expression vector pJD205 (34). Plasmid constructs used as controls in the transfection experiments were all in pJD204 or pJD205 as follows: β actin-luciferase, which was constructed using the human β actin cDNA as a promoter; and RSV-luciferase, which was constructed using the entire LTR from the Rous Sarcoma virus (RSV). These control constructs were generous gifts of S. Langer and M. Ostrowski (Duke Medical Center, Durham, NC), and have been described elsewhere (35). Control experiments were first performed to ensure that luciferase activity could not be detected in cells transfected with the luciferase plasmid pJD205 lacking a promoter.

**Transient Transfection Assays.** CH12-LBK cells were transfected by the DEAE-dextran procedure of Fujita et al. (36). Briefly, 2 × 10⁷ cells/transfection were incubated at 37°C for 90 min in 1 ml Tris-buffered saline, pH 7.4, with 10–25 μg of DNA and 400 μg of DEAE-dextran (Pharmacia LKB Biotechnology, Inc.). DMSO was added to a 10% final concentration and the cells were shocked at 25°C for 3 min, washed, and resuspended at 4 × 10⁵ cells/ml in DMEM-FCS. After 2 h, the cells were divided and half of the cells were stimulated with LPS. The unstimulated and LPS-stimulated cells were harvested after a further 24 h of culture. Luciferase assays were performed according to the method of DeWet et al. (34). Briefly, extracts were prepared by lysing cells in 100 mM KH₂PO₄, pH 7.4, containing 1% NP-40. After the addition of 6 mM ATP and 18 mM Mg²⁺, 100 μl of 0.25 μM D-luciferin (Sigma Chem. Co., St. Louis, MO) was added and peak luminescence was measured for 10 s at 25°C using a luminometer (Berthold Biolumat, Nashua, NH).

**Measurement of Antibody Secretion.** Antibody secretion was measured either by a plaque-forming cell (pfc) assay (29) or determined by ELISA as described elsewhere (36a). For the ELISA, supernatants were collected and antibody was directly quantitated or, alternatively, cells were harvested, washed extensively, and recultured in fresh media. After 6 h, supernatants were collected and the quantity of antibody secreted over this time interval (which has been found to be at a constant rate) was then measured.

**Results**

**Expression of MMTV during B Cell Ontogeny.** In earlier work, we examined the expression of MMTV in several mature (surface Ig⁺) B lymphocytes, and found that they all constitutively expressed MMTV transcripts. We therefore examined the expression of MMTV in B cell lines that represented earlier stages in B cell ontogeny to determine if MMTV expression was restricted to mature B cells. In Fig. 1, results using representative early B lineage cell lines, including NFS-70, a cell in the pro-B cell stage (lane a), NFS-25, a cell line with early pre-B characteristics (lane b), and ABE-8.1/2, a prototypic pre-B cell line (lane c), are shown. MMTV transcripts could be detected in all of these cells at levels similar to constitutive levels in a mammary cell line, MMT (lanes f and g), and in CH12-LBK cells, a mature B cell line (lanes d and e). These results suggest that MMTV is expressed very early in the B lymphocyte differentiation pathway. As previously shown (17), MMTV transcript levels were significantly upregulated after LPS stimulation of the inducible cell line,

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**Figure 1.** MMTV transcript in cells of the B lineage is quantitatively similar to levels of expression in mammary cells. RNA (20 μg/lane) was extracted from various cells and subjected to Northern blot analysis. Blots were probed with the MMTV probe pA8 (up) or actin (bottom). B lineage cell lines include: pro-B cell line NFS-70 (a); pre-B cell lines NFS-25 (b), and ABE-8.1/2 (c); mature B cell line CH12-LBK, either unstimulated (d) or LPS stimulated (e). MMTV expression in the mammary cell line MMT was monitored before (f) and after (g) culture for 24 h with LPS. Non-B lineage hematopoietic cells included: T cell lymphomas R1.1 (h) and BW5147 (i); myelomonocytic cell line WEHI-3 (j); monocye/macrophage lines RAW309.Cr1 (k), and IC-21 (l).
Table 1. MMTV Expression in B lineage Cells

| Differentiation stage | Cells tested | Expression* |
|-----------------------|--------------|-------------|
| B cell lines          |              |             |
| Pro-B                 | NFS-70       | All positive|
| Pre-B                 | 70Z/3, 2E8, NFS-25, ABE-8.1/2, 1A9, NFS-5, 15-79-2 | All positive |
| Mature B              | BCL-1, NFS-1, CH1, CH31, CH27, WEHI-231, A20, CH12, LBK, CH33 | All positive |
| Myelomas, hybridomas* |              | Negative, or only weakly positive |
| Normal B lineage cells|              |             |
| Pro-B                 | Whitlock/Witte (SCID cells)§ | All positive |
| Pre-B/Pre-B           | Whitlock/Witte (BALB/c cells)§ | All positive |
| Mature B§             | B10.A; B6; BALB/c; DBA/2; CBA |             |

* Expression levels were determined by Northern blot analysis.
§ From reference 17.
§ RNA was prepared from non-adherent cells from these cultures. Northern blots were provided by P. Kincade.
§ LPS-activated spleen B cells.

CH12-LBK. In total, we have assayed MMTV expression in a panel of 34 B cell lines representing various stages of the B cell developmental pathway. MMTV transcripts have been detected in all of them, with the sole exception of myeloma/hybridoma cells (Table 1). The lack of expression in myeloma cells is perhaps not surprising, since these cells fail to express a number of genes expressed in other B lineage cells, including mb-1 (37) and MHC class II (38).

To determine if MMTV is expressed in the normal counterparts of early B progenitors, we probed Northern blots (kindly provided by Dr. P. Kincade) containing RNA from B lineage cells isolated from Whitlock-Witte cultures (39) of normal and SCID bone marrow cells, the latter providing an enriched source of pro-B cells. We found MMTV transcripts in RNA from both cultures (not shown), indicating that normal pro-B and pre-B cells are also capable of expressing MMTV transcripts.

We next asked if MMTV transcripts could be detected in cells other than B cells in the hemopoietic lineage. In contrast with the uniform expression of MMTV transcripts in B cells, only one non-B cell line in which MMTV sequences were not amplified, WEHI-3 (lane j), showed significant levels of MMTV transcripts (Fig. 1). This cell line is believed to represent an early hematopoietic cell and it is possible that at this early stage some plasticity allows for the expression of some lineage restricted genes. MMTV transcripts were not detected in three cell lines representing mature stages of the monocyte/macrophage lineage, RAW309 and IC-21 (Fig. 1, lanes k and l) and RAW264, nor in NK cell lines (not shown). MMTV transcripts were not evident in representative T cell lymphomas (Fig. 1, lanes h and i), although we have found that by using very high specific activity probes and by exposing Northern blots for extended periods of time, low levels of MMTV can be detected in some T cell lines.

Expression of MMTV in Normal Tissues. MMTV transcripts are not detectable in most normal tissues (1), including spleen and thymus (see Fig. 2). Based on our observations that MMTV transcripts are expressed at high levels in LPS-stimulated normal B cells (18), we asked if MMTV transcripts were constitutively expressed in normal resting B cells or were expressed only after LPS stimulation (Fig. 2). MMTV transcripts could not be detected in RNA from unstimulated B cells, but were detectable in RNA from LPS-stimulated cells. This was not due to lack of RNA, since both actin and CHO-B transcripts could be detected in all lanes. The lack of MMTV RNA in resting B lymphocytes explains the failure to detect MMTV transcripts in splenic tissue. However, MMTV-specific sequences can be detected in RNA from normal spleen cells using PCR (not shown). Whether this represents very low basal levels of expression in resting B cells, or MMTV transcripts in the small percentage of activated B cells present in the spleen, is not clear. MMTV transcripts are readily detected in activated B cells from a number of independent mouse strains (Table 1).

RNA from T cells stimulated with Con A and IL-2 also contained detectable MMTV transcripts, but at much lower levels than B cells (Fig. 2). The fact that we can detect MMTV transcripts in normal T cells and some T cell lines in low
Transcriptional Regulation of MMTV in B Cells. MMTV expression in B cells could be regulated by at least two mechanisms. Endogenous MMTV proviral loci could be regulated by positional effects, in which integration into chromosomal regions that are transcriptionally active in B cells allows MMTV expression. Alternatively, promoter elements within the MMTV gene itself, in part or in whole, could regulate transcription in B cells. As a means to approach these alternatives, two kinds of experiments were carried out. First, we determined the methylation status of the endogenous loci in B cells as an indication of chromatin configuration, as it has been shown that MMTV loci are heavily methylated in tissues, such as kidney or liver (40), in which they are not expressed. Second, we determined whether the MMTV LTR could act as an independent promoter capable of activating a heterologous gene in B cells.

In the inducible B cell line, CH12, the expressed MMTV transcripts come primarily from the Mtv-9 locus (17), although by PCR analysis either or both of the other loci present in the genome, Mtv-8 and Mtv-17, are expressed at low levels (22). It is possible that only the highly expressed Mtv-9 proviral locus was integrated in the vicinity of a transcriptionally active B cell–specific gene. In fact, Mtv-9 is located on the same chromosome as the IgH locus (chromosome 12), although it is >20 cM removed (41). To determine if the Mtv-9 locus exhibits greater evidence for demethylation than Mtv-8 and Mtv-17, genomic DNA from unstimulated or LPS-stimulated CH12-LBK cells was first digested with EcoRI and then with one of the methylation sensitive enzymes, CfoI or HpaII.
EcoRI-digested genomic DNA, when probed with MMTV env-specific probes, reveals a limited number of restriction fragments which can be assigned to the 3' end of the known endogenous MMTV proviruses within the B10.A genome (3). By such an analysis, one can compare the level of methylation of specific proviral loci in B cells (Fig. 3, lanes b and c) with that of a fully methylated tissue, such as kidney (lane a). In Fig. 3, we show that all three proviral loci were found to be demethylated with respect to one methylation-sensitive enzyme, CfoI. With a second enzyme, HpaII, partial demethylation of all three loci was evident, but additional demethylation occurred upon LPS stimulation, particularly apparent in the Mtv-9 locus (compare lanes c and b). Especially noteworthy is the demethylation of Mtv-8, which to our knowledge, has never been shown to be demethylated in any tissue, including mammary tissue (42). In other experiments, MMTV loci in genomic DNA from normal unstimulated as well as LPS-stimulated B cells, and DNA from T cell lines were also found to be demethylated (not shown). We conclude that demethylation does not strictly correlate with high level transcription. It is therefore unlikely that the integration site alone can account for MMTV expression, although it is possible that the levels or ontogeny of expression of individual proviral loci may be influenced by positional events.

We next determined if the LTR from a cloned endogenous MMTV provirus, Mtv-9 (17), acted as an inducible promoter in B cells. We subcloned the Mtv-9 LTR as the only promoter/enhancer motif driving a luciferase reporter gene (34), and transiently transfected this construct into CH12-LBK cells. MMTV transcripts are upregulated 10-20-fold after stimulation of these cells (see Fig. 1). If the MMTV LTR acts as a promoter/enhancer motif in B cells, a significant increase in luciferase activity should be evident in LPS-stimulated cells compared with unstimulated cells. After transfection, LBK cells were divided into two flasks and either left unstimulated or stimulated with LPS for 24 h. As controls, we included luciferase constructs using a β-actin promoter and an unrelated LTR from RSV. As shown in Fig. 4, in three separate experiments, the Mtv-9 LTR behaved as an independent and inducible promoter, causing a 10-20-fold induction in luciferase expression in LPS-stimulated compared with unstimulated CH12-LBK cells. In contrast, although the RSV LTR and the β-actin constructs were transfected into the same cells into which the Mtv-9 LTR construct was transfected, neither of these control constructs were induced in LPS-stimulated compared with unstimulated cells to any significant extent (<2x). This was despite the fact that the Mtv-9 promoter was the weakest of the three promoters. Compared with actin, the strongest promoter, Mtv-9 was 10-30-fold weaker in the luciferase assay. We conclude that the inducible effect on the MMTV LTR was specific.

Stimulation of MMTV Transcript Levels in Inducible B Lineage Cell Lines. In transgenic mice carrying hybrid MMTV LTR promoter constructs coupled to various protooncogenes, including c-myc, high levels of transgene expression or the development of tumors occur in several tissues, among them salivary gland, kidney, spleen, and mammary epithelia (43-45). Each of these tissues contains secretory cells. Given our data that the MMTV LTR can act as an inducible promoter in a B cell line that can be stimulated to secrete antibody, we were curious if stimulation of MMTV expression correlated with an inducible secretory phenotype in B cells. We therefore compared the ability of MMTV to be stimulated in several B cell lines with their ability to be induced to secrete antibody. For this analysis, we tested the B cell lymphomas CH12, BCL1, A20, CH1, and two sublines of WEHI-231. CH12 cells differentiate in response to IL-2 and LPS, and not IL-2 (36a), and only IL-5 and LPS stimulated increased MMTV expression (Fig. 5). A similar correlation between the ability to stimulate MMTV levels and antibody secretion was found in BCL1 cells. The subline of BCL1 used can be stimulated to secrete antibody by IL-2 and IL-5, but not LPS (46), and MMTV transcripts were also strongly induced by IL-5 and IL-2, but not by LPS (not shown). Moreover, in A20 cells,
MMTV transcript levels are upregulated in CH12 cells only by those stimuli that induce antibody secretion. CH12 cells were cultured alone (−), with LPS, IL-2, IL-5, or IL-2 and IL-5 for 48 h, and the stimulation of transcript levels or antibody secretion, as measured by plaque assay, was determined. (A) RNA was extracted and assayed for the indicated transcripts by Northern blot analysis. (B) Results of the plaque assay, shown as the percentage of the viable recovered cells that secreted sufficient antibody to be detected as a pfc.

The data suggested a possible correlation between the ability to upregulate MMTV transcripts and the ability to induce antibody secretion. The most striking example of this correlation is illustrated using two sublines of WEHI-231, which behave differently in response to LPS. One (WEHI-231a) constitutively secretes low levels of polymeric IgM antibody and is not further induced by LPS, while the second (WEHI-231b) does not secrete detectable levels of antibody unless stimulated with LPS (Fig. 6). Although both sublines express constitutive levels of MMTV transcripts, these transcripts are only upregulated in response to LPS in the WEHI-231b, the cell line that can be induced to secrete antibody (lanes a and b vs. c and d). Taken together, these results support the hypothesis that inducible MMTV expression correlates with inducible, but not constitutive, antibody secretion.

During B cell differentiation, two transcriptional factors, NF-κB and OTF-2, are closely associated with high level expression of Ig (32, 48). Both of these factors are upregulated during B cell differentiation, and increases in the activity of these two factors could influence MMTV expression. Several
cell lines and levels of MMTV do not correlate with NF-κB or OTF-2, with the addition of the OTF-2 probe. In the pre-B cell lines, only LBK cells already had upregulated μ and γ transcripts before LPS stimulation. Probes that were used were the same as those described in Fig. 6, with the addition of the OTF-2 probe. In the pre-B cell lines, only 70Z/3 showed clear evidence of differentiation in response to LPS. CH12-LBK cells already had upregulated μ and κ transcripts before LPS stimulation, while MMTV transcripts (and antibody secretion) were upregulated after LPS stimulation.

Figure 7. MMTV transcripts are not upregulated in stimulated pre-B cell lines and levels of MMTV do not correlate with NF-κB or OTF-2 levels. RNA was extracted from pre-B cell lines (NFS-25, 70Z/3, and 15-79-2) or the mature B cell line CH12-LBK (LBK) that were cultured alone (−) or in the presence of LPS (+) and subjected to Northern blot analysis. Probes that were used were the same as those described in Fig. 6, with the addition of the OTF-2 probe. In the pre-B cell lines, only 70Z/3 showed clear evidence of differentiation in response to LPS. CH12-LBK cells already had upregulated μ and κ transcripts before LPS stimulation, while MMTV transcripts (and antibody secretion) were upregulated after LPS stimulation.

Discussion

The results demonstrate that MMTV proviral transcripts are expressed in all cells of the B lineage, except myelomas, and that the levels of expression are not quantitatively different from the steady state levels found in a mammary epithelial cell line. MMTV transcripts are detectable throughout ontogeny and can be detected in cells representing the pro- and pre-B cell stages as well as their normal counterparts. This implies that the factor(s) involved in constitutive MMTV expression is activated very early after commitment of progenitor cells to the B lineage, but our data suggest that these factors are not NF-κB or OTF-2.

Normal B cells that have been stimulated with LPS express easily detectable MMTV transcripts. However, MMTV expression is detectable in unstimulated cells only by PCR. This is of interest based on the recent demonstration that MMTV genes encode superantigens (25, 27, 28). A number of investigators have shown that superantigens are expressed either in immunogenic form by B cells only after stimulation, or that stimulation enhances superantigen activity (21, 51–54). The failure to detect significant amounts of MMTV transcripts in unstimulated splenic B cells suggests that the reduced immunogenicity of these cells reflects limiting levels of the MMTV-encoded superantigens. It is interesting that superantigens are not expressed by macrophages (51, 55) and it is striking that we failed to detect MMTV transcripts in cells representing the monocyte/macrophage lineage. Some T cells appear to express low levels of MMTV transcripts, but the relationship between expression in these cells and the ability to induce tolerance to Mls-1+ with CD8+ T cells (56) is not known.

The LTR of the expressed endogenous Mtv-9 gene can serve as an LPS-inducible promoter in B lymphocytes, as measured in transient transfection assays (Fig. 4). Similar results were obtained using the LTR from the exogenous C3H virus (not shown). This is the first evidence that we are aware of in which an intact MMTV LTR can be shown to act as a

viral LTRs have functional NF-κB binding sites (49), and OTF-1 has already been demonstrated, in conjunction with steroid hormone receptors, to bind and activate transcription from the MMTV LTR in fibroblasts (9). Indeed, the MMTV LTR has a potential NF-κB site at position −570 relative to the cap site (9/10 nucleotide homology), as well as several overlapping functional octamer motifs at −57, (7/8 homology) (9, 17).

To investigate whether the presence or absence of NF-κB and OTF-2 could be correlated with the differential expression of MMTV transcripts in B cells, we assayed for MMTV expression in a panel of four different B cell lines that represented early pre-B to mature B cell phenotypes. These cells were either left unstimulated or stimulated with LPS before analysis. The activation of κ transcription was taken as a readout for NF-κB activity (48), while OTF-2 levels were measured directly by Northern blot analysis. The results are shown in Fig. 7, and clearly demonstrate that NF-κB and OTF-2 are not sufficient for upregulated expression of MMTV in B lineage cells. In three B lineage cells that represent different stages of the pre-B cell development pathway, MMTV transcripts were not upregulated by LPS, indicating that each of these cells lacks certain required factors. The early pre-B cell NFS-25 does not express γ, μ, or OTF-2 transcripts, and yet MMTV transcripts are present, suggesting that the constitutive levels of MMTV transcription do not require these transcription factors. Activation of NF-κB, as evidenced by κ transcription,
promoter independent of glucocorticoid stimulation. These results support our earlier data which demonstrated that the expression of endogenous MMTV in B cells occurred without GR-GRE interactions, as shown by the failure of the glucocorticoid receptor antagonist, RU486, to inhibit constitutive and LPS-inducible expression of MMTV transcripts (18). The ability of the MMTV LTR to act as an independent promoter in B cells is reasonable evidence that local chromatin configuration and positional effects cannot alone account for the expression of endogenous MMTV loci. However, we cannot rule out the possibility that transcriptionally active genes around one or more of the MMTV proviral loci may contribute to the expressed levels of MMTV. There is evidence demonstrating that active genes can affect the chromosomal configuration of other genes at a distance (57, 58), and at least some MMTV proviruses are closely linked to B cell-specific genes (1, 3, 40). Thus, positional effects could explain why in a cell line only one MMTV locus is expressed at high levels, such as in the case of Mtv-9 expression in CH12 cells (17), despite the obvious demethylation of the other loci, and may explain why the Mls superantigens but not Etc-1 are expressed in unstimulated splenic B cells (59). However, to explain the B cell-specific expression of these and other MMTV loci by strictly a positional effect, one would have to propose that each provirus is integrated near transcriptionally active B cell-specific genes. Since there are >20 proviral loci (3), this seems unlikely. Furthermore, we demonstrate here that the inducible expression of MMTV results from the activity of its own promoter.

MMTV transcripts can be upregulated with the same stimuli that induce differentiation of B cell lines into antibody-secreting cells (Figs. 5 and 6). MMTV levels were upregulated in B cell lines that could be stimulated to differentiate into antibody-secreting cells, while a mature B cell line that could not be further induced to secrete antibody, as well as an inducible pre-B cell line, failed to upregulate MMTV (Figs. 6 and 7). NF-kB and OTF-2, both transcription factors highly expressed in many pre-B and B cells (32, 48), are not sufficient for inducible MMTV expression, as demonstrated using pre-B cell lines (Fig. 7). We have found that OTF-2 levels are high in mature B cells, regardless of MMTV inducibility (unpublished results). However, while MMTV transcripts are upregulated by the same stimuli that induce B cell differentiation, we have previously shown that the upregulated expression of MMTV is clearly independent of the transcriptional up-regulation of Ig genes in stimulated B lymphocytes (18). Thus, other perhaps novel B cell-specific nuclear factors appear to be responsible for the induction of MMTV transcripts in differentiating B cells. These factors are activated during differentiation, and our data strongly implicate an inducible secretory phenotype as the differentiative stage at which MMTV is stimulated at maximum levels in the B cell lineage. Such factors may be of functional significance in the regulation of non-Ig genes that are required for B cell differentiation. Moreover, since there appears to be at least some correlation between the ability to express MMTV at high levels and secretory capacity in other tissues (see Results), it is possible that some of the host transcription factors used by MMTV in these various tissues, including B cells, are at least partially overlapping.

It is difficult to understand the involvement of MMTV in establishment of the T cell repertoire. One might argue that T cells capable of stimulating viral activation are deleted as a normal consequence of T cell repertoire formation. However, in the case of MMTV, this seems unlikely to be of significant selective advantage, since mammary tumors rarely form until well into reproductive age (1). Our data suggest an alternative hypothesis, that the role of MMTV gene products as superantigens reflects the evolution of an adaptive use of the immune system in the life cycle of infectious MMTV (60). MMTV is transmitted horizontally from the milk of lactating female mice of infected strains into the gut of nursing offspring. Lymphoid cells have long been postulated to be involved in the transport of the virus from the gut to other locations in the body (1), and B cells may become infected by virus transferred via milk to the neonate. However, our data indicate that high level expression of MMTV genes is upregulated only after B cell activation, and it is possible that viral replication in B cells is initiated only at this time. The virus may have developed a mechanism to ensure that infected B cells are stimulated with high frequency by producing a product that is mitogenic for a large fraction of T cells, based not on T cell specificity, but on TCR Vβ usage. This, in turn, would result in the activation of the infected B cell. Because the factors required for high level MMTV transcription are present primarily in differentiating and not resting B cells, the data imply that MMTV may indirectly stimulate its own transcription, presumably also increasing its own replicative potential. Production of infectious viruses, migration to mammary tissue, and consequent infection of mammary epithelia then occur. The activation of the virus in mammary tissue as a result of hormonal stimulation during lactation then reinitiates the viral life cycle (1).

How does this relate to the effects of endogenous proviruses on the T cell repertoire? Because the majority of endogenous proviruses are defective and do not produce infectious particles (1, 3), it is likely that the virus captured the use of the immune system in its own infectious life cycle long before the first virus became established in the murine germ line. Many of these integrated proviruses may have retained their ability to stimulate T cells and to be upregulated in B cells, both properties of the highly conserved viral LTR (25, 27, 28, and this report). Because B cells expressing superantigens encoded by these endogenous proviruses are found in the thymus at times critical for T cell development (53, 54), these viruses assumed a new role, namely deletion of self-reactive T cells. The fact that this function has been retained through evolution suggests that it could have been adapted by the host as an important component in the selection of the T cell repertoire. We favor an alternative hypothesis: the deletion of large components of T cells based on Vβ usage may have no consequence for the development of a full and functioning T cell repertoire, as long as too many proviruses capable of deleting different subsets of T cells are not present in the genome of any given animal. The fact that T cells with a given antigen specificity may express different...
Vβ alleles is consistent with this latter possibility. Moreover, mice totally lacking known endogenous MMTV have been identified (61, 62), suggesting that the effects of the endogenous superantigens on the developing T cell repertoire are unnecessary for survival in the wild.

One of the predictions of this hypothesis is that not all species will necessarily contain endogenous superantigens, since they are of little direct consequence for T cell repertoire selection. To take an extreme view, it is possible that the only species that have endogenous superantigens are those that house viruses that have, during their evolution as infectious agents, taken advantage of the capacity of T cells and B cells to stimulate each other as a necessary component of their own life cycle.

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