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Multiple Endogenous Xenotropic and Mink Cell Focus-Forming Murine Leukemia Virus-Related Transcripts Are Induced by Polyclonal Immune Activators

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Northern (RNA) analyses were used to study the kinetics of induction of endogenous mink cell focus-forming (MCF) and xenotropic murine leukemia virus (MuLV)-related sequences in NFS and C57BL/6 mice injected with the polyclonal immune activators lipopolysaccharide (LPS), concanavalin A, and 8-bromoguanosine. All three mitogens induced 8.4-, 7.2-, 3.0-, and 1.8-kilobase (kb) MCF-related transcripts coordinately in the spleens of injected mice. Xenotropic MuLV-related expression was also rapidly induced in spleens by the three polyclonal immune activators, but in a noncoordinate manner: a distinct set of transcripts with different kinetics of expression was induced by each mitogen. MCF-related induction after LPS injection was both rapid and sustained; it began within 30 min and persisted for at least 8 days in the spleens of both NFS and C57BL/6 mice. LPS also caused prolonged induction of xenotropic transcripts in spleens of C57BL/6 but not NFS mice. The *gld* mutation, which causes polyclonal immune activation, induced 8.4-, 10.0-, and 13-kb MCF-related transcripts in C3H/HeJ mice without altering expression of 7.2-, 5.6-, 4.0-, 3.0-, or 1.8-kb MCF-related transcripts. The data demonstrate that individual endogenous MuLV-related transcripts can be induced coordinately or independently and suggest that expression of these transcripts is linked to early stages of lymphocyte activation.

Infectious murine leukemia viruses (MuLVs) can be divided into three classes with different host ranges. Ecotropic retroviruses infect only mouse cells; xenotropic retroviruses infect only nonmouse cells; and polytropic, or mink cell focus-forming (MCF), viruses infect both mouse and nonmouse cells. Genomic line DNA from all inbred mouse strains contains multiple copies of MuLV-related sequences, the majority of which are replication defective (29). While infectious ecotropic and xenotropic retroviruses are present in the germ lines of many inbred mice, infectious MCF MuLVs do not preexist in the genome. Instead, MCF viruses arise via recombination between endogenous ecotropic, xenotropiclike, and MCF-related sequences (4, 14, 35). Endogenous MCF-related transcripts can be distinguished from recombinant MCF viruses; only the former have a 190-base-pair (bp) cellular DNA insert in their long terminal repeat (LTR) (16).

Expression of endogenous MuLV-related sequences can be induced by activation of the immune system. In vitro stimulation of spleen cells with the B-cell mitogen lipopolysaccharide (LPS) induces expression of xenotropic virus and gp70 protein in many mouse strains (6, 24). This induction was detected in B cells after 2 to 3 days of stimulation and was blocked by preventing immunoglobulin M expression with anti-immunoglobulin M or by inhibiting DNA synthesis (1, 31, 34). These in vitro studies suggested that LPS-induced xenotropic expression requires B-cell proliferation and differentiation. It is unknown whether MCF-related expression is also induced by B-cell mitogens. T-cell mitogens, such as concanavalin A (ConA), generally have not been found to induce significant retroviral expression (1, 31), although increased T-cell membrane MuLV-related gp70 has been reported (37).

We have found a novel endogenous 8.4-kilobase (kb) MCF-related transcript which is highly expressed in autoimmune but not nonautoimmune mouse strains (19). Polyclonal B-cell activation is a characteristic of autoimmune mouse strains (10, 17; Y. Ishigatsubo, A. D. Steinberg, and D. M. Klinman, Eur. J. Immunol., in press). To investigate the relationship between immune activation and endogenous MuLV-related expression, we examined endogenous xenotropic and MCF-related expression in nonautoimmune mice injected with the T-cell mitogen ConA and the B-cell mitogens LPS and 8-bromoguanosine (8-BG). While LPS appears to bind to a membrane receptor, 8-BG acts intracellularly to induce polyclonal B-lymphocyte proliferation and differentiation (39). We also studied the effects of the *gld* mutation, which induces polyclonal B-cell activation and autoimmunity in C3H/HeJ mice (28; Ishigatsubo et al., in press), on MuLV-related expression.

MATERIALS AND METHODS

Mice. C57BL/6, BALB/c, C3H/HeJ, and C3H/HeJ-*gld*/*gld* mice were obtained from Jackson Laboratory (Bar Harbor, Maine), and NFS mice were from the Frederick Cancer Research Facility (Frederick, Md.).

Polyclonal immune activators. ConA type IV-S, 8-BG, and *Salmonella typhimurium* LPS were purchased from Sigma Chemical Co. (St. Louis, Mo.). Protein-free LPS was generously provided by D. Morrison (University of Kansas, Kansas City). All activators were diluted in sterile phosphate-buffered saline and injected intraperitoneally in a volume of 0.1 ml.

Probes and Northern (RNA) blot hybridizations. MCF_{env} and $xeno_{env}$ oligonucleotide probes consist of 16 and 30 bp of the MCF and xenotropic 5′ *env* regions, respectively, and

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hybridize specifically to endogenous MCF and xenotropic transcripts (15, 20). Transcripts detected with these probes are herein referred to as MCF-related and xenotropic transcripts, respectively. Mouse beta-2-microglobulin is a 0.6-kb cDNA generously provided by F. Mushinski (National Institutes of Health). Glyceraldehyde-3-phosphate dehydrogenase is a rat 1.3-kb cDNA obtained from M. Piechaczyk (25). LTR_{190} (previously called $B\text{-}34_{162}$ [15]) is an *Alu*I-to-*Bgl*II fragment from the U3 region of the B-34 MuLV LTR and consists of 162 bp of the 190-bp cellular DNA insert characteristic of endogenous MCF-related LTRs (16). The *pol* gene probe is a 1.4-kb *Xho*I-to-*Hpa*II fragment of the AKR 623 ecotropic MuLV DNA, and the *gag* gene probe is a 0.9-kb *Bgl*II-to-*Bam*HI fragment of AKR MCF 247 MuLV DNA (20). Ecotropic, xenotropic, and MCF MuLV total RNAs were included with each blot for molecular size determination and hybridization specificity (15).

Mouse tissues were homogenized with a polytron for 1 min at high speed in 4 M guanidine thiocyanate (5). RNA was separated by centrifugation through a 5.7 M cesium chloride cushion, followed by suspension and extraction with phenol-chloroform (1:1) and chloroform. Poly(A)$^+$ RNA was selected by passage over oligo(dT) columns (2). RNA was denatured in 50% formamide–1.3% formaldehyde–20 mM MOPS (morpholinepropanesulfonic acid) (pH 7.0) at 55°C for 15 min and then size fractionated in a 1% agarose–20 mM MOPS (pH 7.0)–0.7% formaldehyde gel containing ethidium bromide. Custom-made long narrow gel combs were manufactured by Bethesda Research Laboratories, Inc. (Bethesda, Md.). RNA was blotted onto nitrocellulose paper (Schleicher & Schuell, Inc., Keene, N.H.), and complete transfer was documented by UV photography before and after blotting. Blots probed with oligonucleotide probes were prehybridized at 45°C in 50 mM Tris (pH 8)–10% dextran sulfate–1% sodium dodecyl sulfate (SDS)–1 mg of yeast RNA (type X-S; Sigma) per ml–1 M NaCl, hybridized at 45°C in the same mixture containing 3×10^6 cpm of oligonucleotide end labeled with polynucleotide kinase (Pharmacia, Inc., Piscataway, N.J.) per ml and [γ-^{32}P]ATP (>5,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and washed at 45°C for MCF_{env} or 42°C for $xeno_{env}$ for 15 min in 6× SSC (1× is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS and for 30 min in 1× SSC–1% SDS. Northern blot hybridizations with nonoligonucleotide probes were performed by prehybridization at 42°C in 50% formamide–5× SSC–5× Denhardt solution–0.1% SDS, hybridization at 42°C in 50% formamide–5× SSPE–1× Denhardt solution–10% dextran sulfate–0.1% SDS–10^6 cpm of [α-^{32}P]dCTP probe labeled with a random priming kit (Pharmacia) per ml, and washing once at room temperature and once at 55°C in 2× SSC–0.1% SDS for 15 min and four times at 55°C in 0.2× SSC–0.1% SDS for 15 min each. Stripping of probes was achieved by washing in 0.01× SSPE (1× is 0.15 M NaCl, 0.01 M sodium diphosphate, 0.001 M EDTA)–0.1% SDS at 70°C for 10 to 30 min in an agitating water bath and was confirmed by autoradiography. Probes were purified by centrifugation through G-25 Sephadex (oligonucleotides) or G-50 Sephadex (nonoligonucleotides) columns from 5 Prime-3 Prime Inc. (Philadelphia, Pa.). Autoradiography was performed at −70°C with Kodak XAR film in the presence of Cronex Lightning-Plus intensifying screens.

RESULTS

Kinetics of induction of endogenous MCF-related and xenotropic transcripts in NFS mice by LPS. NFS mice were injected intraperitoneally with LPS; at various times afterward, RNA was extracted from tissues and Northern blot analyses were performed. Within 4 h of LPS injection, the spleen contained increased amounts of 7.2-, 3.0-, and 1.8-kb MCF-related transcripts as well as newly detectable 8.4-kb MCF-related RNA (Fig. 1). All four transcripts were induced within 2 h in a subsequent experiment (data not shown). These four transcripts hybridized with the LTR_{190} probe (data not shown) and therefore contained the 190-bp DNA sequences characteristic of endogenous MCF-related LTRs (16). The 8.4-kb transcripts were no longer detectable 8 h after injection, but the shorter transcripts continued to be highly expressed for at least 8 days (Fig. 1).

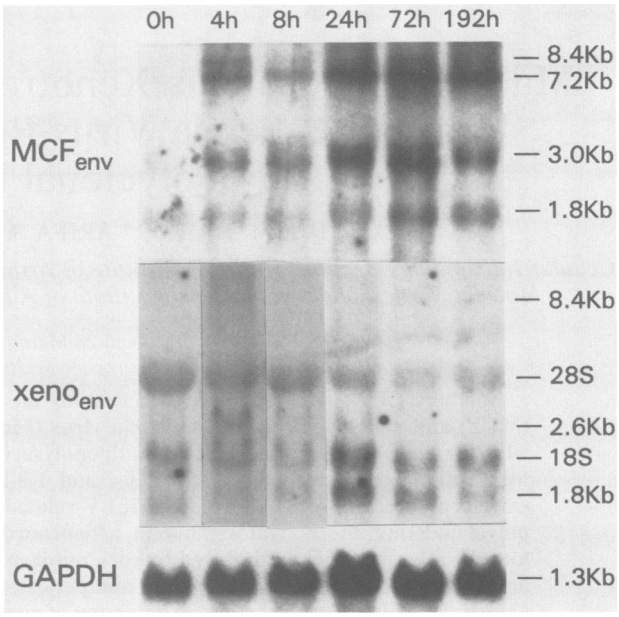

FIG. 1. Induction of spleen retroviral expression by LPS. Four-month-old male NFS mice were injected intraperitoneally with 50 μg of *S. typhimurium* LPS. At the indicated times, spleen tissues from two to four mice were processed into RNA. Total RNA (20 μg) was used for Northern blot analyses. The same blot was stripped and reprobed serially with MCF_{env}, $xeno_{env}$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Induction of faint 8.4-kb spleen xenotropic RNA was observed at 4 h, but not at later time points. A 2.6-kb transcript was detectable from 4 to 24 h after LPS injection, and a 1.8-kb xenotropic transcript was present at relatively constant levels throughout the experiment (Fig. 1).

LPS injection also increased MCF-related RNA in the NFS mouse thymus (Fig. 2). At 4 h, expression of 7.2- and 3.0-kb MCF-related sequences was greatly increased; however, by 24 h this expression had returned to base line. There was no apparent induction of 8.4-kb MCF-related RNA nor of xenotropic expression (Fig. 2). We cannot rule out a direct effect of LPS on thymocytes; however, mediators of the acute-phase response induced by LPS may well be responsible for the observed thymic induction.

Liver 7.2- and 3.0-kb MCF-related transcripts were increased by 4 h, peaked at 8 h, and returned to base line within 24 h after LPS injection (Fig. 2). No 8.4-kb MCF-related transcripts were detected. A 2.6-kb xenotropic transcript was induced in the liver at 4 and 8 h after LPS injection (Fig. 2).

ConA and 8-BG induce MuLV-related expression in NFS mice. Intraperitoneal injection of the T-cell mitogen ConA

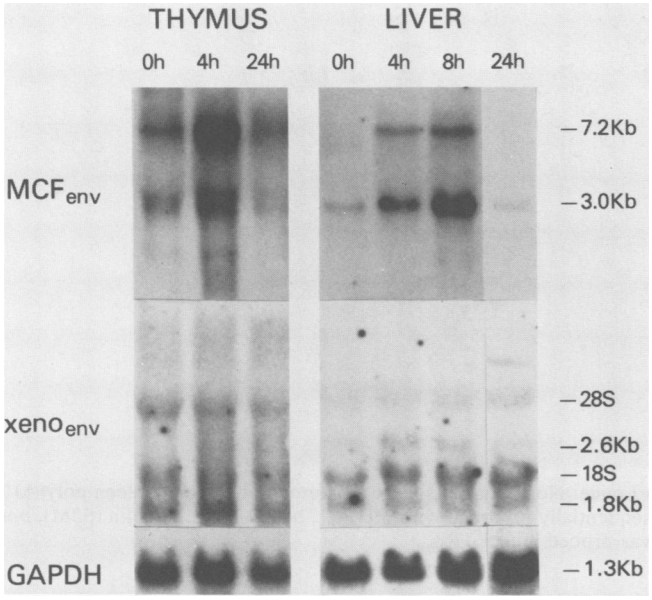

FIG. 2. Induction of thymus and liver retroviral expression by LPS. Total thymus and liver RNAs (20 μg) from the same NFS mice studied in Fig. 1 were probed with MCF$_{env}$, xeno$_{env}$, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Time in hours after LPS injection is indicated above each lane.

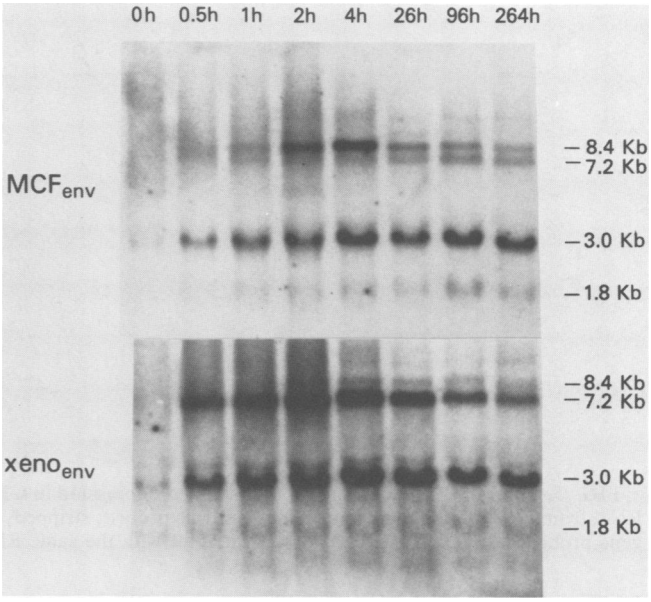

FIG. 4. Retroviral induction in C57BL/6 mice by LPS. Four-month-old male C57BL/6 mice were injected intraperitoneally with 50 μg of LPS. At the times shown, total RNA was prepared from the spleens of individual mice, and 20 μg was used for Northern blot analysis. The same blot was probed serially with MCF$_{env}$ and xeno$_{env}$. Cross hybridization of xeno$_{env}$ with rRNA was reduced by washing at 45°C. Ethidium bromide staining was used to confirm that comparable amounts of RNA were present in all lanes.

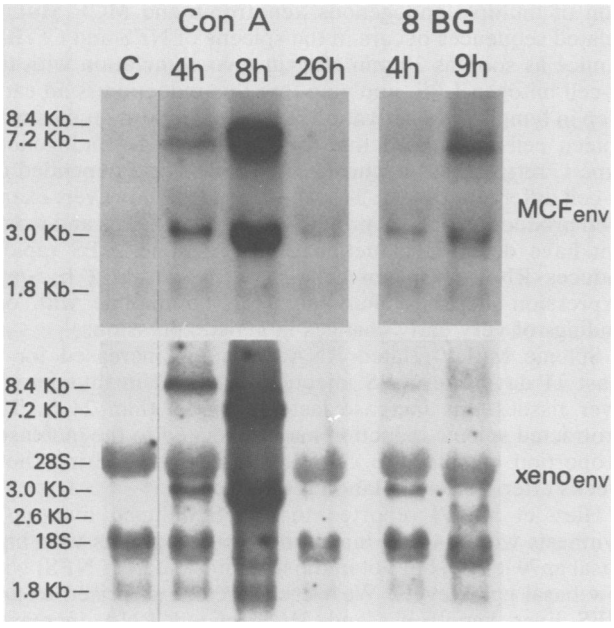

FIG. 3. Induction of splenic retroviral expression by ConA and 8-BG. Five-month-old male NFS mice were injected intraperitoneally with 100 μg of ConA or 75 μg of 8-BG. At the indicated number of hours after injection (the control, C, is 0 h), total RNA was prepared from spleen tissues of individual mice, and 20 μg was used for Northern blot analyses. All lanes are from the same blot, which was probed with MCF$_{env}$, then stripped and reprobed with xeno$_{env}$. Ethidium bromide staining was used to confirm that equal amounts of RNA were loaded in each lane.

led to a rapid increase in splenic levels of 8.4-, 7.2-, 3.0-, and 1.8-kb MCF-related RNA (Fig. 3). In contrast to the sustained increase in splenic MCF-related transcripts after LPS injection, levels of MCF-related RNA returned to base line within 26 h after ConA injection (Fig. 3). ConA injection also caused marked induction of xenotropic RNA: 8.4- and 3.0-kb bands were seen at 4 and 8 h; large amounts of a 7.2-kb transcript were present at 8 h (Fig. 3).

MCF-related induction after 8-BG injection of NFS mice was similar to that seen after ConA or LPS: 8.4-, 7.2-, 3.0-, and 1.8-kb transcripts appeared within 4 h (Fig. 3). At 4 h after 8-BG injection, xenotropic 3.0-kb and faint 8.4-kb transcripts were induced; at 9 h, xenotropic 2.6-kb and faint 8.4-kb transcripts were detected (Fig. 3).

MCF-related and xenotropic induction by LPS in C57BL/6 mice. Within 30 min of LPS injection, C57BL/6 mice expressed increased amounts of 8.4-, 7.2-, 3.0-, and 1.8-kb MCF-related transcripts (Fig. 4). Expression of 8.4-kb MCF-related RNA peaked at 4 h and then decreased, while expression of the shorter transcripts peaked later and continued at a more constant level for the entire 11 days of this experiment. Expression of xenotropic 7.2- and 3.0-kb transcripts was also increased by 30 min and persisted for 11 days (Fig. 4). A faint 8.4-kb xenotropic band was detectable from 2 h to 11 days after LPS injection. There was no apparent induction of 1.8-kb xenotropic RNA. In a separate experiment, 8.4-, 7.2-, 3.0-, and 1.8-kb MCF-related transcripts and 2.6- and 8.4-kb xenotropic transcripts were increased in spleens of BALB/c mice within 2 h of LPS injection (data not shown).

***gld* mutation induces several MCF-related transcripts.** The *gld* mutation arose spontaneously in C3H/HeJ mice; it causes polyclonal B-cell activation with autoantibody production, splenomegaly, and massive lymphadenopathy (28;

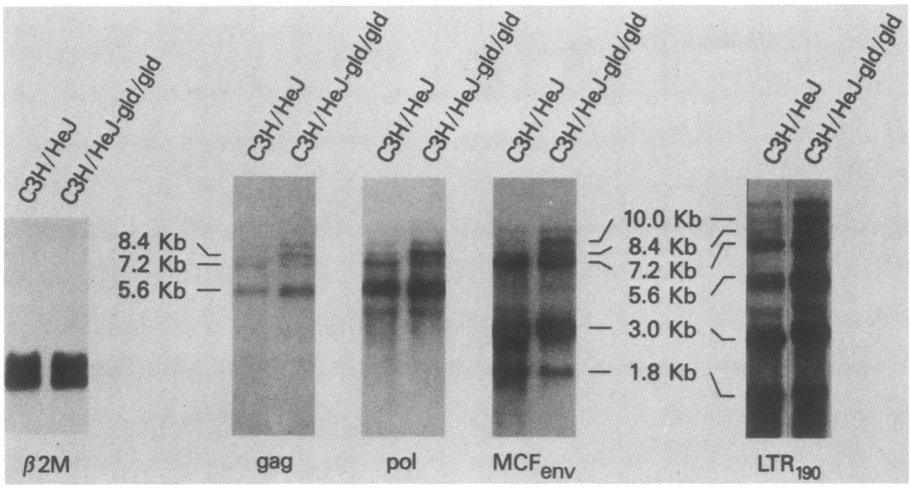

FIG. 5. Effect of *gld* mutation on MCF-related expression in C3H/HeJ mice. Northern blots were performed on 5 μg of spleen poly(A)$^+$ RNA from 5-month-old male mice. The filter was probed, stripped, and sequentially reprobed with MCF_{env}, beta-2-microglobulin (β2M), *pol* gene probe, and *gag* gene probe. A separate blot with the same RNA was probed with LTR_{190}.

Ishigatsubo et al., in press). To determine whether constitutive (as opposed to exogenously induced) polyclonal immune activation would alter endogenous MuLV-related expression, we studied C3H/HeJ and congenic C3H/HeJ-*gld/gld* mice. Both strains had extremely low levels of xenotropic 3.0- and 1.8-kb *env*-related transcripts and no ecotropic MuLV-related expression (data not shown). Therefore, any other bands detected with MuLV-specific probes should be transcribed from endogenous MCF-related sequences.

Poly(A)$^+$ RNA from C3H/HeJ-*gld/gld* spleens contained eight different MuLV-related transcripts, five of which were expressed at similar levels in C3H/HeJ (Fig. 5; data summarized in Table 1). All eight transcripts hybridized with LTR_{190}, a probe specific for the 190-bp sequence characteristic of endogenous MCF-related LTRs, thus confirming the MCF relatedness of these transcripts. The 8.4-, 10.0-, and 13-kb MCF-related transcripts were induced in C3H/HeJ-*gld/gld* and faintly detected in C3H/HeJ (Fig. 5, far right panel). Many different subgenomic MuLV-related transcripts have been reported previously (15, 19, 20, 22), but supergenomic transcripts have not. Non-MuLV supergenomic transcripts have been reported (8, 12).

Hybridization of 8.4-, 10.0-, and 13-kb MCF-related RNAs to MCF_{env}, LTR_{190}, and *pol* and *gag* MuLV-specific probes suggested that these transcripts have complete retroviral genomes (Fig. 5; Table 1). The 7.2-kb RNA does not contain p15E sequences and could therefore be distinguished from the larger transcripts which do (data not shown).

TABLE 1. Reactivity of MuLV DNA probes with C3H/HeJ-*gld/gld* RNA

Transcript size (kb)	Probe			
	gag	*pol*	*env*	LTR_{190}
13	?[a]	+	+	+
10	+	+	+	+
8.4	+	+	+	+
7.2	+	+	+	+
5.6	+	+	−	+
4.0	−	+	?	+
3.0	−	−	+	+
1.8	−	−	+	+

[a] ?, Possible faint band.

C3H/HeJ mice have a membrane defect resulting in unresponsiveness to the mitogenic effects of LPS (11). To determine whether this defect would prevent the LPS-induced increase in endogenous MuLV-related RNA, we injected C3H/HeJ mice with protein-free LPS. There was no change in xenotropic or MCF-related RNA levels (data not shown), demonstrating that the cellular pathways involved in the responses to LPS and *gld* are at least partly separate.

DISCUSSION

The data presented in this report demonstrate that induction of multiple endogenous xenotropic and MCF MuLV-related sequences occurs in the spleens of NFS and C57BL/6 mice as soon as 30 min after in vivo stimulation with the B-cell mitogen LPS, implying that this induction is an early step in lymphocyte activation. Previous in vitro studies with spleen cells suggested that LPS induction of endogenous type C retroviruses occurred after 2 days and depended on B-cell differentiation (1, 34). Those studies, however, examined production of gp70 protein or infectious virus and would not have detected induction of RNA alone. LPS rapidly induces RNA in spleen cells in vitro; within 1 h c-*myc* expression increases markedly (13), compatible with our findings of very early changes in gene expression.

Splenic MuLV-related RNA remained increased for at least 11 days after LPS injection, whereas in thymus and liver tissues this increase lasted for less than 24 h. The protracted splenic induction may be related to the increased proportion of spleen B cells in the S phase during the 2 weeks after LPS stimulation (26).

Hara et al. (7) reported that LPS induced liver gp70 synthesis within 4 h of injection in mouse strains with high basal gp70 levels, but not in those strains (such as NFS) with low basal gp70 levels. We were therefore surprised to find NFS liver xenotropic and MCF-related RNA increased within 4 h after LPS injection. Presumably, these transcripts do not encode gp70 protein detectable by the radioimmunoassay used in earlier studies.

Full-length (8.4-kb) xenotropic and MCF-related transcripts were induced by mitogen in both NFS and C57BL/6 mice, but do not necessarily encode protein or infectious virus. Indeed, there is no known endogenous infectious MCF virus, and the inducible locus for infectious xenotropic

virus, Bxv-1, is present in C57BL/6 but not NFS mice (9, 18). While infectious virus is not induced by LPS in NFS mice, reverse transcriptase is, compatible with the expression of endogenous defective retroviral sequences (33).

The regulation of expression of endogenous MuLV-related sequences is largely unknown. The GV-1 locus is important in the coordinate regulation of several endogenous MuLV-related sequences (21). We observed coordinate induction of four MCF *env*-related transcripts (8.4, 7.2, 3.0, and 1.8 kb) by the mitogens LPS, ConA, and 8-BG. Continued expression of these transcripts after LPS induction was somewhat noncoordinate: expression of the 8.4-kb MCF-related transcript peaked early (within 4 h) and then decreased, whereas the shorter transcripts continued to be highly expressed for more than a week in the spleens of both NFS and C57BL/6 mice. In contrast, induction of xenotropic expression was noncoordinate: in NFS mice, each mitogen induced a different combination of transcripts and had different kinetics of expression. LPS induced xenotropic 8.4- and 2.6-kb transcripts, ConA induced 8.4-, 7.2-, and 3.0-kb transcripts, and 8-BG induced 8.4-, 3.0-, and 2.6-kb xenotropic RNAs. These data suggest that multiple factors regulate expression of endogenous MuLV-related sequences. The various combinations of xenotropic transcripts induced by the three mitogens may reflect the different mechanisms of mitogenesis.

The mechanism by which mitogens induce MuLV-related transcripts is unclear and may differ for different transcripts or mouse strains. Some of the differences in expression between the different strains could be due to variations in their content of endogenous retroviruslike sequences. Although NFS mice have only one endogenous xenotropic *env*-related sequence (9), five different xenotropic *env*-related transcripts were seen after mitogen induction (8.4, 7.2, 3.0, 2.6, and 1.8 kb). Possible explanations for this include mitogen-induced transcriptional readthrough, recombinational events, and changes in RNA splicing or processing. Variable RNA splicing has been reported in C3H/HeJ-*gld*/*gld* mice (30, 36) and could account for the additional high-molecular-weight MCF-related transcripts observed in those mice.

Another possible mechanism of induction is suggested by the chromosomal location of MuLV-related sequences. Many xenotropic and MCF-related sequences are located near genes encoding lymphocyte antigens (*Thy-1*, *Ly-2*, *Lyb-2*, *Ly-6*, *Ly-7*, *Ly-10*, *Ly-22*, and the immunoglobulin heavy chain and kappa light chain) (3, 27, 38). Mitogen-induced activation of these genes could secondarily result in induction of nearby MuLV-related sequences via a *cis* effect.

Endogenous MuLV-related LTRs have promoter activity (23) and an enhancer region capable of binding to cellular *trans*-acting factors (20, 32). Therefore, activation of endogenous LTRs by mitogen could cause transcription of nearby genes, possibly with important immunologic effects.

The biologic significance of endogenous MuLV-related expression after polyclonal immune activation is unknown. We have recently reported a novel 8.4-kb endogenous MCF-related transcript which is highly expressed in autoimmune but not nonautoimmune mouse strains (19). As discussed above, an 8.4-kb MCF-related transcript was induced in nonautoimmune mice by polyclonal immune stimulators, but its expression was briefer than that of the shorter transcripts, suggesting that the 8.4-kb RNA is subject to different regulatory factors. Of course, it is unclear whether these different 8.4-kb MCF-related transcripts are transcribed from the same locus or are unrelated. It is possible that the constitutive expression of an 8.4-kb MCF-related transcript in autoimmune mouse strains contributes to the polyclonal activation which is a feature of their disease (10, 17). Alternatively, this expression may be a secondary epiphenomenon. Studies of the regulation of expression of endogenous retroviral sequences may provide insights into normal cellular activation mechanisms and abnormalities associated with autoimmune disease.

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