We have identified a gene whose expression appears to be associated with a late stage in the differentiation of B lymphocytes into antibody secreting cells, as shown by using the inducible B cell lymphoma, CH12. Restriction mapping and partial sequencing of a cDNA clone isolated by subtraction analysis demonstrated that the clone, SC34, represents an envelope (env) gene transcript of a mouse mammary tumor virus (MMTV). In CH12 cells and in normal B cells, levels of MMTV RNA were increased after stimulation with LPS. The env transcript was the predominant MMTV RNA species and increased more dramatically than did levels of the genomic transcript. In differentiating CH12 cells, env transcripts increased as much as 20-fold above levels found in replicating, antibody nonsecreting CH12 cells. The major increase in expression appeared to be associated with B cell differentiation and not replication. By Southern blot analysis, only bands characteristic of endogenous proviruses were found in CH12, indicating that viral sequences were not amplified in this cell line. Restriction mapping indicated that the SC34 cDNA clone was a product of the Mtv-9 locus. Mtv-9 previously was shown to encode a complete MMTV provirus on chromosome 12, on which Ig heavy chain genes also are located. Increases in MMTV transcripts followed distinct kinetics and were quantitatively different from changes in immunoglobulin gene products. The expression of env RNA appears to more accurately reflect differentiation to antibody secretion in CH12 cells than does the expression of immunoglobulin gene transcripts.

The primary function of B lymphocytes is to secrete antibody. Upon stimulation, B cells enter cell cycle, replicate, and at least some members of the responding clones differentiate into high rate antibody secreting cells (1-3). Considerable insights in understanding the initial events of this process have been gained during the past few years. The identity of "triggering receptors" on the B cell membrane, the nature of the signals transduced across B cell membranes after ligand-receptor binding, the factors (cytokines) involved in promoting B cell growth and differentiation, and some of the genetic events involved in the growth process have been subjects of intense study (4-14). Proteins that increase or decrease during B cell differentiation have been identified (15, 16), but their roles in this process remain unknown. Clearly, receptors for growth and differentiation factors, regulatory proteins, and proteins involved in the transport of Ig must be regulated during B cell activation and differentiation.

Despite these advances in understanding B cell activation, the molecular events that are primarily associated with the differentiative process remain obscure. This is, in large part, due to the inability of separating the proliferative and differentiative stages of B cell activation in most experimental systems. Therefore, with the exception of several well characterized genes, such as those encoding immunoglobulins and J chain, which are transcriptionally induced and post-transcriptionally regulated during B cell differentiation (7, 9, 11, 17, 18), the identity of other genes that are regulated remain unknown.

As a first step in understanding the differentiative process in B cells, we have set out to identify and characterize non-Ig genes that are induced during B cell differentiation. A B cell lymphoma, CH12, serves as a useful model for these studies because it can be stimulated to high rate antibody secretion in the presence of LPS (16, 19). We have isolated a subclone of CH12 that differentiates in response to LPS without significantly altering its rate of proliferation. Therefore, changes in gene expression after LPS stimulation more likely reflect differentiative and not proliferative events. We have prepared a subtractive probe to detect genes that increase in abundance during CH12 differentiation, and used it to screen a cDNA library from LPS-stimulated CH12 cells (20). Candidate differentiation-specific clones were isolated and screened for expression using RNA from the CH12 subclone.

In this study, we report the isolation and characterization of one cDNA clone, SC34, which represents a gene product that is highly inducible during the differentiation of CH12 cells and normal B cells. SC34 is derived from a
transcript from a MMTV envelope gene. Southern blot analysis indicated that MMTV genes are not amplified in the CH12 cell line, therefore indicating that all transcripts are from endogenous proviruses. Restriction map analysis revealed that the SC34 cDNA clone originated from the Mtv-9 locus, an endogenous MMTV proviral gene located on chromosome 12 (21).

MATERIALS AND METHODS

Materials. B10.A mice were bred in our mouse colony by using breeding stock from Jackson Laboratories (Bar Harbor, ME). FBS was purchased from J.R. Scientific (Woodland, CA). All media and salt solutions for lymphocyte cultures were prepared using endotoxin free water from an Ultra 70 system (Zenon Environmental Inc., Burlington, Ontario). Restrictions enzymes, Klenow, DNA polymerase and DNA ligase were from Boehringer Mannheim Biochemicals (Indianapolis, IN) and New England Biolabs (Beverly, MA); amu reverse transcriptase was purchased from Life Sciences (St. Petersburg, FL), a-thi-DATP and dCTP were from Amersham (Arlington Heights, IL).

Lymphocyte cultures. The characterization of the CH12 B cell lymphoma previously was reported (5, 7, 16). The experiments reported here were carried out by using CH12-LBK, an in vitro subclone of the original ascites form of the CH12 lymphoma. The derivation of this subclone is described elsewhere (21). CH12-LBK (hereafter referred to as CH12) cells were maintained in DMEM supplemented as described (16) and which contained 10% FBS and 5 × 10^{-2}M 2-ME. They were stimulated at cell concentrations of 5 × 10^4/ml with 50 μg/ml LPS Escherichia coli 055:B5 (Difco, Detroit, MI) in cultures containing 5 × 10^{-2}M 2-ME. Antibody secreting cells were enumerated by using a plaque forming cell assay with BrSRBC as indicator cells. A 5% suspension of SRBC was incubated (37°C, 30 min) with 100 μg/ml bromelin (Sigma, St. Louis, MO) and washed three times before use. BrSRBC provide a more sensitive indicator of antibody secretion by CH12 cells than do untreated SRBC as used in previous studies (5, 7).

Lymphocytes were enriched from spleens of B10.A mice by removal of erythrocytes by hypotonic lysis and depletion of T cells with mixtures of anti-Thy-1 and anti-Ly-1 antibodies and complement as described (22). B cells were stimulated with LPS by using conditions described for CH12.

Isolation of RNA. Total cellular RNA was extracted using the lithium chloride/urea method of Auffray and Rougé (23). Polyadenylated RNA was selected using oligo (dT)-cellulose (Pharmacia, Piscataway, NJ) chromatography (24).

Construction of cDNA libraries. cDNA libraries were constructed from mRNA of nonstimulated and LPS-stimulated cells from the in vivo CH12 cell line. Single-stranded cDNA was synthesized by oligo (dT) priming of poly A RNA by using amu reverse transcriptase in the presence of actinomycin D (25). The second strand was synthesized by using the Okamaya and Berg (26) method using RNase H and E. coli DNA polymerase (27). The ds cDNA was treated with RNase A and H and methylated and flushed loaded with T4 DNA polymerase and EcoRI linkers then were added, digested with EcoRI and the cDNA was fractionated on a BioGel A-50M column. cDNA inserts (0.2 to >2.0 kb in size) were ligated into the plage vector a gt10 (imm™5527) (27) and packaged. Each a gt10 library had a titer of 5 × 10^6 independent clones. Approximately 0.7 × 10^3 clones were amplified in E. coli C600 hflA150 and were used for screening.

Generation of cDNA subtraction probe. As outlined in Figure 1, a cDNA subtraction probe was generated through solution hybridization using methods similar to those described by Davis et al. (25) and Kavathas et al. (28). A-thi-DATP labeled first-strand cDNA was synthesized from poly A RNA by using oligo (dT) priming of poly A RNA by using amu reverse transcriptase in the presence of actinomycin D (25). The second strand was synthesized by using the Okamaya and Berg (26) method using RNase H and E. coli DNA polymerase (27). The ds cDNA was treated with RNase A and H and methylated and flushed loaded with T4 DNA polymerase and EcoRI linkers then were added, digested with EcoRI and the cDNA was fractionated on a BioGel A-50M column. cDNA inserts (0.2 to >2.0 kb in size) were ligated into the plage vector a gt10 (imm™5527) (27) and packaged. Each a gt10 library had a titer of 5 × 10^6 independent clones. Approximately 0.7 × 10^3 clones were amplified in E. coli C600 hflA150 and were used for screening.

RESULTS

CH12 cells differentiate in response to LPS. When cultured in the presence of LPS, CH12 cells differentiate into high-rate antibody secreting cells with as many as 50% of the cells secreting antibody as determined by plaque assay. Figure 2 shows the results of two combined experiments in which the time course of growth and secretion after addition of LPS to the cells is depicted. We have consistently observed that stimulation with LPS does not increase the doubling time of CH12 cells, but it does significantly increase Ig secretion. This indicates that the primary effect of LPS on these cells is to induce differentiation without concomitant changes in proliferation.

Isolation of the SC34 clone. Approximately 12,000 recombinant clones in the activated CH12 cDNA library were screened with the cDNA subtractive probe (Figure
A large fraction of the clones identified by the subtractive probe were expected to be cDNA clones encoding Ig \( \mu \) or \( \kappa \). After LPS-stimulation, the abundance of Ig transcripts is increased eight- to 10-fold in the in vivo CH12 cell line, the line used for construction of the cDNA libraries and for production of the subtractive probe. Indeed, clones representing Ig comprise at least 10% of the clones in the cDNA library constructed from mRNA from LPS-stimulated CH12 cells, whereas fewer than 1.5% of the clones in the nonactivated library were identified as representing Ig (20). Therefore, candidate clones were probed for \( \mu \) and \( \kappa \) in order to reduce the possibility of isolating subtraction clones that represented Ig transcripts. Among 85 differentiation specific clones, 17 clones were isolated and could be classified into four groups based on DNA cross-hybridization (20). Clone SC34 was the unique member of one of these four groups.

Figure 3 shows an RNA dot blot analysis using the insert of the clone SC34 (\(-1.5 \text{ kb}\)) to probe RNA from LPS-stimulated or LPS-stimulated CH12 cells isolated at varying times of culture. An increased signal was detected using RNA from LPS-stimulated cells but not from unstimulated cells, suggesting that RNA detected by this probe increased in abundance during differentiation. The increase appeared to be maximal within 24 h of culture after LPS stimulation.

A plasmid probe of SC34 (pSC34) (Fig. 4), constructed by subcloning the SC34 insert into the EcoRI site in pBR328, was used to probe a Northern blot containing RNA from LPS-stimulated and unstimulated CH12 cells. Two RNA species were detected, one a 3.8 kb RNA, and a second transcript of approximately 9.0 kb that was visible on longer exposures (Fig. 4, panel b). The expression of both RNA species was increased in LPS stimulated cells. This increase was specific, as shown by the use of an actin control. Interestingly, the change in expression of the two RNA species was not the same (Table I). Whereas the 9.0 kb transcript increased 3.7-fold upon stimulation, the 3.8 kb RNA increased 12-fold. These same blots were probed with Ig \( \mu \) and \( \kappa \) probes for comparison. When Ig probes were used on the Northern blot shown in Figure 4, the increases were less than fourfold, similar to those of the 9.0 kb RNA species detected by pSC34 and far less than the increase in the 3.8 kb RNA species (Table I). It should be noted that, in unstimulated cells from the CH12 subclone used in these studies, constitutive levels of both \( \mu \) and \( \kappa \) RNA are increased relative to levels in the in vivo line, and LPS induces changes in expression that are less dramatic than in the in vivo line (20).

SC34 encodes a mouse mammary tumor virus env gene product. In Figure 5, the SC34 insert was subcloned into M13 and a sequence from one end of the clone was determined to allow a homology search of known sequences to be performed. The sequence revealed 97 to 98% identity with two different MMTV provirus env sequences reported by Redmond and Dickson (43) and Salmons et al. (44). These two proviruses are encoded by the Mtv-2 and Mtv-8 proviral loci, defined according to the recently revised nomenclature (45). To confirm the identity of the SC34 insert, a restriction map analysis was performed (Fig. 5). The restriction sites found in SC34 were identical to those in the same region of the Mtv-2 MMTV proviral gene except that SC34 lacked a BamHI site found in the Mtv-2 sequence. This BamHI site is missing in all MMTV proviruses encoded in the
Figure 4. Northern blot analysis of RNA from nonactivated (NA) and
LPS-stimulated (A) CH12 cells. RNA (30 µg) was resolved on a 1% formal-
aldehyde-agarose gel and transferred for probing with the pSC34 probe
(panels a and b). An actin probe (pHF-1) was used to control for equal
loading and equivalence of transfer of RNA in the two lanes (panel c).
Panels a and b are different exposures of the same blot. Panel a shows
an overnight exposure (−20°C) and panel b shows the same blot overex-
posed (three days, −70°C) to highlight the upper bands. An RNA ladder
was resolved (three days, −70°C) to highlight the upper bands. An RNA ladder
was scanned with a densitometer and quantitated for intensity. The results shown
for generation of the C57BL/10 mouse strain (46), the strain of
origin from which the MMTV proviruses of the CH12
donor strain (B10. H-2 4-p/Wts) were derived (see also
Fig. 7). From this analysis, we conclude that SC34 en-
codes a MMTV proviral env sequence, and that the two
RNA transcripts that hybridize with the pSC34 probe on
Northern blotting represent the full length genomic trans-
script (35S) and the 24S processed env transcript.

Expression of the env gene in normal B cells. We next
asked whether the inducible expression of MMTV tran-
scripts was limited to the CH12 cell line or whether their
expression was also a property of normal B cells. To
address this issue, B cells from B10.A mice were stimu-
lated for varying periods of time with LPS. RNA was
extracted, and Northern blot analysis was performed
using the pSC34 insert. Figure 6 shows that the pSC34
insert hybridizes with a 3.7–3.8 kb RNA species (Fig.
6A), confirming that MMTV env sequences were ex-
pressed in normal B cells. Genomic length RNA was not
detectable in these blots although they have been de-
tected in other Northern blots (data not shown). The level of
env transcripts increased steadily between days 1 and
3 of culture (Fig. 6B). We have been unable to quantitate
changes in stimulated vs unstimulated B cells because of
the difficulty in obtaining sufficient amounts of RNA
from resting (G1) B cells from the spleen. Indeed, we have
been unable to determine whether MMTV transcripts are
present in unstimulated normal B cells. Nevertheless, our
data demonstrate that a) MMTV transcripts are expressed
in normal splenic B cells following LPS stimulation, and
b) the steady state levels of these transcripts increase
during differentiation.

SC34 is encoded by the endogenous Mtv-9 provirus.
Three endogenous MMTV proviruses are transmitted in
the germ line of B10.A mice: Mtv-2, Mtv-9, and Mtv-17
(46, 47). Some tumors of T cell origin, however, express
exogenous or amplified viruses that account for the
expression of MMTV transcripts (48–50). To determine
whether the expression of MMTV RNA in CH12 cells was
due to the transcription of one or more germine genes,
or if there was a contribution from amplified viral se-
quences, Southern blot analysis was carried out. Genom-
ic DNA from CH12 cells was digested with the restric-
tion endonucleases EcoRI or BamHI and subjected to
Southern blot analysis for comparison with DNA from
normal tissue of B10.A mice. In each set of restrictions,
the pSC34 probe hybridized with three identical bands in DNA isolated from CH12 cells and from normal kidney, and each could be assigned to one of the known endogenous proviruses (Figure 7). Thus, EcoRI fragments of sizes 6.7 kb, 8.3 kb, and 10.0 kb were detected and corresponded to the 3' ends of the Mtv-8, Mtv-17, and Mtv-9 proviruses, respectively. The BamHI digests could be assigned to the same 3 loci (Figure 7). In other experiments, digestion with HindIII and PstI yielded the expected number of bands (3 and 1, respectively; data not shown). Therefore, only the number and sizes of restriction fragments expected from hybridization with non-amplified endogenous proviruses was observed by using genomic DNA from CH12 cells. “Smearing” of the Southern blots, caused by hybridization of the probe with randomly integrated viruses, as found with the use of these same enzymes and genomic DNA from cells in which viral amplification had occurred (48-50), was not observed. We conclude that 1) the expression of MMTV transcripts in differentiating CH12 cells is caused only by the expression of one or more of the endogenous proviruses, and 2) that the SC34 insert is encoded by one of these endogenous proviral genes.

To identify which of the three loci was responsible for encoding SC34, restriction mapping was performed. Mtv-8, Mtv-9, and Mtv-17 can be distinguished using the enzymes SacI and Hpal (46, 47). The SC34 insert, shown in Figure 8, was found to contain a single SacI site and two Hpal sites. The locations of these sites were consistent with those published by Peters et al. (46) but a discrepancy with the positioning of the 5' Hpal site described by Peterson et al. (47) was observed (not shown). We attribute this difference to inexact positioning of restriction fragments in mapping of genomic DNA in the latter study. The simplest conclusion from these data is that the SC34 insert is a product of the Mtv-9 gene.

Appearance of MMTV env RNA during differentiation. We next determined the rate of appearance of the env transcript after stimulation of CH12 cells with LPS, and compared its increase with changes in expression of Ig transcripts (Fig. 9). Increases were observed within 6 h of stimulation, and maximal env expression was detected within 24 h, accounting for more than a 20-fold increase in expression (bottom panel, Fig. 9). In contrast, only modest increases in Ig μ and κ transcripts were observed and, for both, the largest increases were observed 48 h after stimulation, when maximum numbers of pfc were detected in LPS-stimulated cultures (Fig. 2). Thus, the increase in the expression of the env gene product after LPS stimulation precedes that of Ig genes in this subclone of CH12 cells, and occurs before maximum differentiation of the B cells occurs, as measured by antibody secretion.

DISCUSSION

Transcripts from endogenous MMTV proviral gene(s) are amplified during B cell differentiation. In CH12 cells, increases in the level of MMTV env RNA can be detected within 6 h after addition of LPS, attaining maximal levels approximately 24 h after stimulation. To estimate the amount of env RNA in differentiating CH12 cells, a cDNA library made from mRNA obtained from these cells 48 h after LPS stimulation was probed with a MMTVLTR probe (provided by M. Ostrowski). Approximately 0.2% of the recombinant clones were found to encode MMTV sequences (data not shown). Because maximal levels of MMTV env expression occur earlier than 48 h after stimulation (Fig. 9), more than 0.2% of the RNA in LPS-stimulated cells may constitute env transcripts at peak expression.

Our data are consistent with a strong association of MMTV expression in B cells with their differentiation to antibody secretion, and not with cell proliferation. In CH12 cells, env transcripts rapidly increase in abundance and reach maximal levels around the time of peak antibody secretion, 24 to 48 h after stimulation. It is unclear if env transcripts are expressed at other times during activation. MMTV transcripts are constitutively expressed in unstimulated CH12 cells, albeit to a low level, and it is possible that MMTV genes are activated prior to the commitment of a B cell to differentiation. However, in CH12 cells, a small number (1 to 5%) of cells in the “unstimulated” population secrete antibody. The constitutive expression of env RNA could, therefore, be
due to high levels of expression in these cells, rather than to low levels of expression in all CH12 cells in this population. Similarly, in normal B cells, expression of env transcripts is maximal 48 to 72 h after stimulation (Fig. 6). At this time, polyclonal antibody secretion is maximal, and proliferative capacity of the responding B cells is declining under our culture conditions, again suggesting an association of increased env RNA expression with the differentiative phase. In normal B cells, however, env RNA is detectable within 24 h of LPS stimulation, a time prior to maximal induction of pfc. Although it is possible that MMTV transcripts are expressed prior to differentiation, it is likely that asynchronous differentiation of B cells after LPS stimulation could account for the expression of env transcripts in the early periods following B cell stimulation.

Antigenic or mitogenic stimulation has long been known to induce retroviruses or retroviral products in normal lymphocyte populations in both murine and avian model systems (51–58). In these studies, B cells were the predominant cell type identified as expressing retroviruses or their products. Although the majority of these studies were concerned with the expression of C-type viruses, Tax et al. (58) reported the expression of an
antigen cross-reactive with MMTV gp52 on a small population of B cells in normal mice. Although the identity of the subset of B cells that reacted with the anti-gp52 monoclonal antibody was not determined, it is interesting to speculate that they may have been differentiated B cells. The frequency of cells detected by the anti-gp52 antibody (4 to 6%) is similar in magnitude to the number of differentiated B cells in the spleens of normal mice (59). A correlation between retroviral gene expression and B cell differentiation was documented in the study of Alberto et al. (57), who reported a direct correlation between the expression of murine leukemia viral encoded gp70 on B cell membranes and the presence of cytoplasmic Ig. Similarly, Ewert, Haipern, and colleagues (54–56) showed that maximal expression of retroviruses in avian lymphocytes occurred during the differentiation of B lymphocytes into plasma cells. These studies clearly established a link between the late stages of B cell differentiation and the expression of a C type retroviral gene product. Our studies confirm and extend these observations to MMTV env expression, but further demonstrate that the majority or all of increased retroviral expression occurs after Ig gene transcripts are increased, but prior to maximal differentiation of the B cell to antibody secretion.

The expression of MMTV proviruses is restricted to certain tissues, and this tissue specificity is believed to be conferred by sequences in the LTR (60, 48). In particular, expression of MMTV genes is primarily limited to mammary tissue (61). Clearly, however, other cells can express these genes. A number of T cell lymphomas from C57BL mice (48, 49) and kidney adenocarcinomas from BALB/c mice (60) have been isolated that express MMTV. In both tissues, MMTV proviruses are amplified and the U3 domain of the LTR is altered, either by deletions or substitutions, in the region that confers tissue specificity (48, 60). Expression in these tissues may therefore result from mechanisms distinct from those used by B lymphocytes. By Southern blot analysis, we have no evidence that MMTV proviruses are amplified in CH12. Additional evidence that nonamplified viruses may be expressed in normal lymphoid tissue comes from the work of Kwon and Weissman (48), who observed that phorbol esters stimulated env expression in spleen cells of normal mice. Although the cellular source of env expression in stimulated spleen cells in this study was not determined, our results strongly suggest that at least normal B lymphocytes can express env transcripts, since RNA from LPS-stimulated populations of enriched B cells (Fig. 6) was positive by Northern blot analysis. Thus, B cells, like mammary tissue, may be unique in that expression of MMTV genes is permitted without amplification or structural alterations in the viral LTR. We have not ruled out the possibility that the U3 region of the LTR of Mtv-9 deviates from sequences of other LTRs. The integrity of the U3 region of the Mtv-9 gene is, therefore, currently under investigation. Whether or not MMTV is a "B cell specific" gene, or capable of being expressed in other normal hematopoietic tissues, is also being studied.

The role that MMTV gene products play in B cell differentiation, if any, is unknown. It is curious that many of the endogenous MMTV proviruses, including all three of the proviral genes encoded in the B6/B10 backgrounds, are on chromosomes that encode genes relevant to B cell function. Mtv-8 is closely linked to the kappa light chain loci on chromosome 6 (62, 63); Mtv-9 is encoded on chromosome 12, but is apparently unlinked to the Ig heavy chain loci (21). Mtv-17 is on chromosome 4, closely linked to the Lyb-2 locus (46) that encodes a membrane molecule believed to be involved in B cell activation (64). It is not known whether the chromosomal location of the MMTV proviruses has any functional significance for the B cell. It is possible that these regions of the chromosome are transcriptionally active in stimulated B lymphocytes, thus increasing MMTV expression. However, this seems unlikely in the case of Mtv-9 because it is linked to the Fv-4 locus on chromosome 12, distal to the Ig loci (21), making such putative "local" effects unlikely. Although our data indicate that the Mtv-9 locus is active, it should be noted that we do not yet know if either of the other two germine encoded loci is expressed.

Although MMTV is expressed rapidly in CH12 cells after LPS stimulation, several hours are required for induction, suggesting that a cellular factor(s) is responsible for the activation of the proviral gene. The involvement of trans activating factors in the expression of endogenous MMTV proviruses is not without precedent. In normal mammary tissues, the expression of MMTV proviral genes is regulated by factors that, interestingly, are induced by LPS (65, 66). In B lymphocytes, the relationship of the factors that participate in the activation of MMTV to those factors known to be active during B cell differentiation, such as those that act on the Ig loci (67, 68), is unknown. It is worth noting that, in unstimulated cells from the subclone of CH12 used in the current experiments, μ, κ, J chain mRNA and protein are all expressed in near maximal levels, and the transcripts are increased in abundance only slightly by LPS stimulation (Table 1 and Fig. 9). Thus, whereas the increased expression of env precedes further increases in Ig transcripts, Ig genes already have been induced to a very significant extent. This suggests that at least some of the transcriptional factors that act on Ig genes may be constitutively expressed in CH12 cells. MMTV proviruses may be responsive to these factors but only after a differentiative stimulus, such as LPS, is given to the B cell. Alternatively, LPS may activate other regulatory factors that participate in the differentiative process and that are responsible for transcriptional activation of the MMTV provirus. It seems likely that cis regulating elements, perhaps encoded within the LTR, govern activation of the provirus during B cell differentiation. Such regulatory regions should be useful for the isolation and characterization of cellular factors involved in the late stages of B cell differentiation.

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