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The Coupling between Enhancer Activity and Hypomethylation of κ Immunoglobulin Genes Is Developmentally Regulated

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Previous studies have indicated that immunoglobulin enhancers are essential for establishing transcriptional competence but not for maintaining the activity of constitutively transcribed genes. To understand the basis for this developmental shift away from dependence on enhancer function, we have investigated the relationship between transcriptional activity and methylation status of the immunoglobulin κ light-chain genes (κ genes) in mouse cell lines representing different stages of B-cell maturation. Using pre-B-cell lines in which the level of a critical κ enhancer-binding factor, NF-κB, was controlled by the administration or withdrawal of lipopolysaccharide and plasmacytoma lines that either contain or lack this factor, we studied the properties of endogenous κ genes and of transfected κ genes which were stably integrated into the genomes of these cells. In the pre-B cells, the exogenous (originally unmethylated) κ genes, as well as the endogenous κ genes, were fully methylated and persistently dependent on enhancer function, even after more than 30 generations in a transcriptionally active state. In plasmacytoma cells, the endogenous κ genes were invariably hypomethylated, whereas exogenous κ genes were hypomethylated only in cells that contain NF-κB and are thus permissive for κ enhancer function. These results indicate that the linkage of hypomethylation to enhancer-dependent activation of κ transcription occurs after the pre-B-cell stage of development. The change in methylation status, together with associated changes in chromatin structure, may suffice to eliminate or lessen the importance of the enhancer for the maintenance of the transcriptionally active state.

The importance of enhancer elements in transcriptional regulation of eucaryotic genes is well established, although the mechanism by which these elements exert their effect is still poorly understood. The concept of enhancers rests on experiments in which appropriately modified recombinant genes are transfected into cells and assayed for their transient or stable expression (4). For some genes, such as those encoding the immunoglobulin heavy chains and immunoglobulin κ light chains, enhancer function is clearly required for efficient transcription of transfected genes (7). Thus, it was surprising to find that endogenous immunoglobulin genes continue to be transcribed in plasmacytoma cells when their enhancers are deleted (14, 33, 36) or are rendered nonfunctional by the loss of a critical nuclear protein factor (3). These findings led to the idea that the immunoglobulin enhancers are essential for establishing transcriptional competence but not for maintaining the activity of constitutively transcribed genes. The possibility that other enhancers may also have a transient role in transcriptional regulation was suggested by studies of the simian virus 40 enhancer in temperature-sensitive COS cells (34).

According to this view of enhancer function, the activity of the κ enhancer should be crucial during the transition from pre-B to B cells, when cells shift from the expression of heavy chains alone to the expression of both heavy and light chains. In fact, the enhancer may serve not only to establish transcriptional competence at the κ locus, but also to render the $J_κ$ region accessible to the DNA recombinases that catalyze κ gene rearrangements. Direct evidence implicating the enhancer in the developmental activation of the κ locus was provided by studies of its role in mediating lipopolysaccharide (LPS)-induced precocious κ transcription in a variety of pre-B-cell lines (3, 19). After transcription is established at the κ locus, the importance of the κ enhancer would presumably diminish during further maturation of the B lymphocyte.

What is the mechanism by which immunoglobulin enhancers become increasingly superfluous as B-lymphocyte maturation proceeds? One possibility is that the enhancer function is superseded by developmentally controlled structural alterations of the immunoglobulin genes. Indeed, a previous comparison of constitutively expressed and LPS-induced κ genes indicated that such an alteration might include a propensity for hypomethylation (19, 20). These observations also indicated that enhancer activation alone might not be sufficient to alter the methylation pattern, suggesting that the coupling of such structural alterations to enhancer activity might itself be a developmentally regulated phenomenon.

To gain a deeper understanding of these relationships, we have introduced unmethylated κ genes into cells characteristic of early and late developmental stages and have compared their expression and methylation status with those of the endogenous κ genes under defined conditions of κ enhancer function. We have observed that the endogenous κ locus of a pre-B cell remains fully methylated and dependent on enhancer function for its transcriptional activity, even after it has been maintained in a transcriptionally active state for over 20 successive cell doublings. Moreover, we show that this property is also manifested by a transfected κ gene which is stably integrated into the pre-B-cell genome and which exhibits LPS-inducible (enhancer-dependent) transcription. In contrast, exogenous κ genes do not become detectably methylated after introduction into the genome of a plasmacytoma cell, provided that the cells are permissive for κ enhancer function and, consequently, for the establishment of κ transcription. These results indicate that the

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developmental shift toward reduced dependence on enhancer function is associated with increased coupling between enhancer activity and hypomethylation.

MATERIALS AND METHODS

Origin of the cell lines, culture conditions, transfection protocols, and selection procedures have been previously described or referenced (2, 3, 19). The transfected DNA fragments were as follows: an 8-kilobase (kb) *Bam*HI fragment containing the $V_\kappa 19J_2C_\kappa$ gene (10), inserted either into the *Bam*HI site of pUC18 and linearized at the *Sal*I site or into the *Bam*HI site of pSV2*gpt* (18) in opposite orientation to the simian virus 40 promoter region and linearized at the *Eco*RI site; linearized forms of the construct V21/19 (2); pSV2*neo* (29) linearized at the *Bam*HI site; and pSV2*gpt* linearized at the *Bam*HI site. The procedures for DNA isolation (21), Southern blotting (26), and isolation of nuclear, cytoplasmic and total cell poly(A)$^+$ RNA (11, 16) have been described previously. An earlier method for RNA (Northern) blotting (11) was modified as follows. RNA transfer was to Nytran (Schleicher & Schuell, Inc.); 0.1% sodium PP_i and 1% sodium dodecyl sulfate were present during prehybridization and hybridization; and 1 mg of heparin per ml was substituted for Denhardt solution.

RESULTS

Methylation of the κ locus in a pre-B-cell line after many generations of enhancer-dependent transcription. In a pre-B-cell line, such as the Abelson murine leukemia virus-transformed cell line 3-1, exposure to LPS for a few hours induces transcription of the unrearranged (κ^0) locus and the appearance of a DNase I-hypersensitive site in the κ enhancer region (19). This inductive effect seems to be mediated by a posttranslational modification (possibly phosphorylation by protein kinase C) which enables the nuclear protein NF-κB to bind to and activate the κ enhancer (3, 27). Earlier studies in which cells were examined a few generations after exposure to LPS indicated that the induction is not accompanied by demethylation of sites that are invariably hypomethylated in the constitutively transcribed κ genes of more mature B lymphocytes (19, 20). To determine whether this failure to become hypomethylated would persist over many cell generations, we exposed exponentially growing 3-1 cells to 10 μg of LPS per ml for 23 successive cell doublings (Fig. 1a) and then monitored both κ^0 transcription and the methylation status of the κ^0 locus. As seen in a Northern blot analysis (Fig. 1b), these long-term-treated cells continued to produce the 8-kb transcript that is diagnostic for κ^0 transcription (lane A). Removal of LPS from the culture medium resulted in cessation of κ^0 transcription and loss of the 8-kb transcript due to turnover (lane B), whereas subsequent replenishment of LPS rapidly restored transcription (lane C). These data demonstrate that κ^0 transcription remains strictly enhancer dependent throughout a lengthy exposure to LPS. Analysis of three *Hha*I sites that lie roughly midway between the κ enhancer and the κ^0 promoter elements revealed that these sites have remained fully methylated and hence, refractory to digestion by *Hha*I, even after 23 generations of enhancer-dependent κ^0 transcription (Fig. 1c). In contrast, these same *Hha*I sites were entirely unmethylated in a B-cell lymphoma CH31, in which the κ^0 allele and a productively

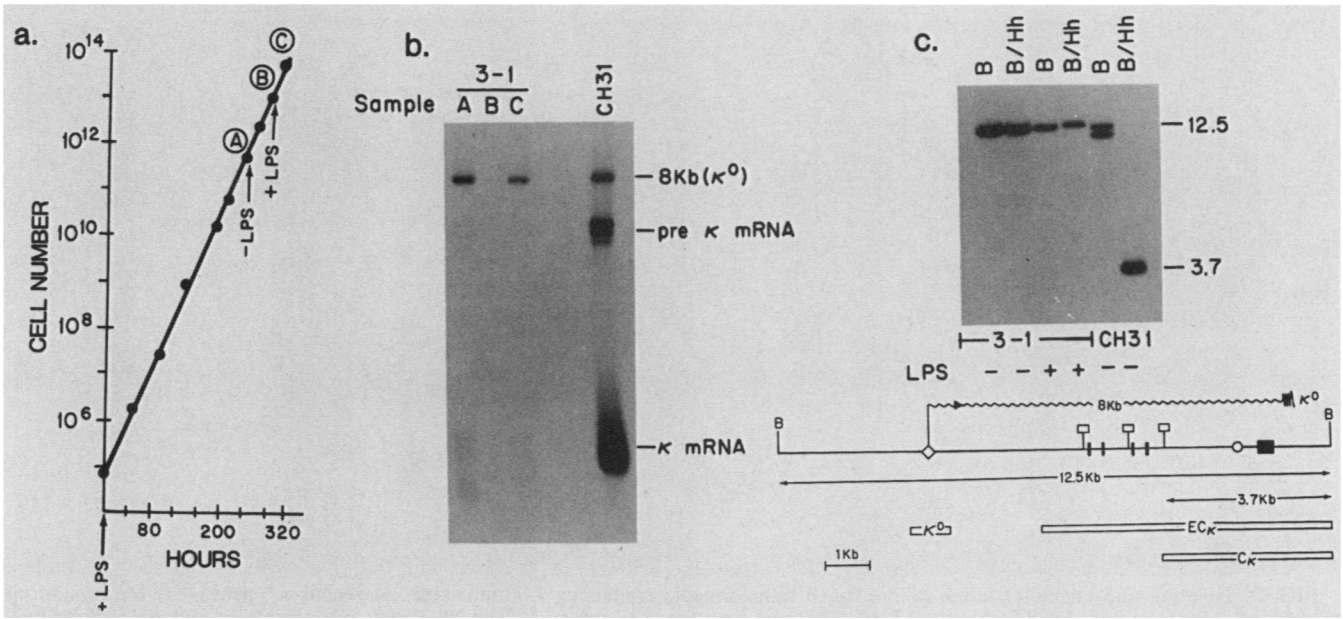

FIG. 1. Analysis of κ RNA transcripts and methylation status of κ loci in an LPS-treated pre-B-cell line (3-1) and B-cell lymphoma cells (CH31). (a) Growth curve of a culture of 3-1 cells maintained for 2 weeks at a concentration of 6×10^4 to 1.8×10^6 cells per ml by periodic dilutions. Except for the period between 238 and 282 h, the medium contained LPS at a concentration of 10 μg/ml. A, B, and C indicate the times at which samples of cells were harvested for RNA and DNA analyses. (b) Northern blot of 5 μg of poly(A)$^+$ RNA from 3-1 cells, harvested at times A, B, and C, and 0.5 μg from CH31 cells, probed with the EC_κ fragment. The positions of the 8-kb κ^0 transcripts and the products of the CH31 κ^+ allele are indicated at the right. (c) Southern blot of DNA from uninduced 3-1 cells (−), LPS-treated 3-1 cells harvested at point A (+), and CH31 cells. DNA was digested with *Bam*HI (B) or *Bam*HI and *Hha*I (B/Hh) and probed with the C_κ fragment. The diagram below depicts the κ^0 locus with J_κ and C_κ exons (▬), the promoter (◇), the enhancer (○), the κ^0 transcript (〰), and the *Hha*I sites (⚲). The κ^0, EC_κ, and C_κ probes are *Eco*RI, *Eco*RI-*Bam*HI, and *Xba*I-*Bam*HI fragments, respectively. Resistance of the 12.5-kb *Bam*HI fragment to *Hha*I digestion indicates that each of the three *Hha*I sites was fully methylated; if these sites were entirely unmethylated, the *Bam*HI-*Hha*I digest yielded a 3.7-kb fragment when analyzed with the C_κ probe and 6.8-, 3.7-, 1.2-, and 0.8-kb fragments when analyzed with the EC_κ probe. Kb, kb.

rearranged (κ^+) allele were both constitutively transcribed (Fig. 1b and c; also data not shown).

Isolation and characterization of pre-B-cell transformants that contain an LPS-inducible exogenous κ gene. To determine whether the relationship between persistent enhancer dependence and sustained methylation is peculiar to the endogenous κ genes of pre-B cells or whether it would also apply to initially unmethylated exogenous κ genes that are introduced into these cells, we carried out a series of transfection experiments with a cloned 8-kb DNA fragment encompassing the κ^+ allele of MPC11 cells, $V_\kappa 19A\text{-}J_2C_\kappa$ (10). Separate DNA fragments, one containing the κ^+ gene and the other containing a selectable marker gene, pSV2*neo*, were cotransfected by electroporation into 3-1 cells or into another line that exhibits LPS-induced transcription of endogenous κ genes, 70Z/3. Pooled samples of G418-resistant transformants were screened for κ^0 gene expression with and without LPS by RNA dot blotting and hybridization to a $V_\kappa 19$-specific probe (Fig. 2a). We obtained stable transformants of both cell lines in which the expression of the introduced κ^+ gene was strongly LPS dependent. After suitable subcloning, one of these LPS-dependent transformants, termed 3-1(T), was selected for detailed study.

A comparative Southern blot analysis of DNA from 3-1(T) and 3-1 cells (Fig. 2b) indicated that a copy of the complete 8-kb κ^+ fragment was integrated at a single locus in the 3-1(T) genome. The 8-kb fragment was seen, in addition to the unrearranged $V_\kappa 19A$ gene and four related members of the $V_\kappa 19$ family, when a *Bam*HI digest was hybridized with the $V_\kappa 19$-specific probe (lane 1 versus 2) or in addition to the 12.5-kb κ^0 fragment when hybridized to the C_κ region probe (lane 5 versus 6). An *Msp*I digest probed for $V_\kappa 19$ sequences revealed a single novel fragment in 3-1(T) (lane 3 versus 4). Since the *Msp*I site in the V region is the only one within the 8-kb *Bam*HI fragment, a single 5′ integration site is indicated. These data, together with additional analyses of *Hin*dIII and *Xba*I digests probed for the 5′ and 3′ ends of the κ^+ gene yielded a map of the integrated gene (Fig. 2b).

Although the foregoing analysis demonstrated that there is a single copy of the κ^+ gene at a unique integration site, the autoradiographic intensities of the 8-kb *Bam*HI fragments were consistently found to be slightly greater than those of the fragments bearing the unrearranged $V_\kappa 19A$ or κ^0 genes. Since 3-1(T) is believed to contain both allelic copies of κ^0, as judged by calibration against single κ allele standards, the comparative intensity of the 8-kb *Bam*HI fragment suggests that the 3-1(T) cells may contain two or possibly three identical copies of the integrated κ^+ gene. Such a situation could arise if the chromosome or chromosomal region bearing the integrated κ^+ gene were preferentially replicated during the initial outgrowth of the 3-1(T) clone. Whatever its origin, this apparent multiplicity was considered to be of no consequence for our present experiments, since identical gene copies should presumably exhibit uniform properties. Within the scope of our analysis (see below), this indeed seems to be the case.

The LPS response of the transfected κ^+ and the endogenous κ^0 genes in 3-1(T) was examined by Northern blot analysis with a suitable set of probes (Fig. 2c). Marked stimulation of transcript production from both genes was

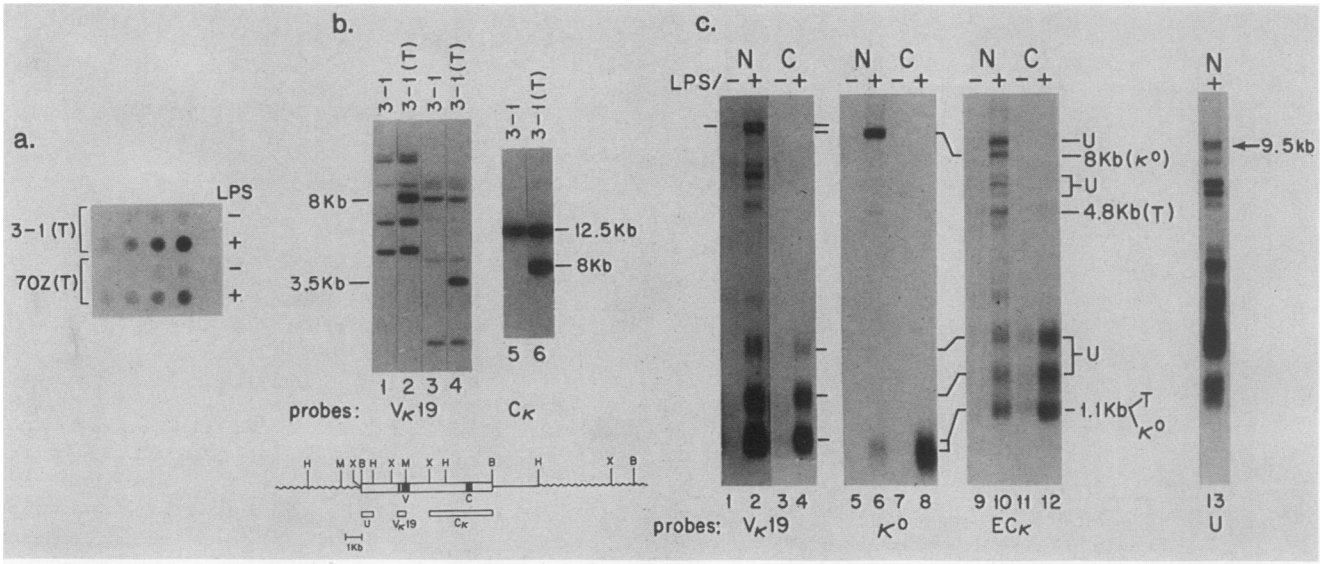

FIG. 2. Isolation and characterization of pre-B-cell transformants containing LPS-inducible exogenous κ^+ genes. (a) Expression of transfected κ genes with (+) and without (−) LPS. G418-resistant transformants of 3-1 and 70Z/3 cells were grown up and divided into two equal portions, one of which was exposed to 10 μg of LPS per ml for 24 h. Total cell RNA was extracted from each sample, dotted onto nitrocellulose sheets (1-, 2-, 4-, and 8-μg portions), and hybridized with the $V_\kappa 19$ probe, which is specific for the transfected κ^+ gene. (b) Southern blot analysis and map of the κ^+ gene in the cloned transformant 3-1(T). DNA from parental 3-1 cells or from 3-1(T) was digested with *Bam*HI (lanes 1, 2, 5, and 6) or *Msp*I (lanes 3 and 4) and analyzed with either the $V_\kappa 19$ or the C_κ probe as indicated. The 8-kb *Bam*HI fragment and the 3.5-kb *Msp*I fragment are derived from the integrated κ^+ gene; the 12.5-kb C_κ-specific fragment contains the endogenous κ^0 gene; the other $V_\kappa 19$-specific fragments contain the unrearranged $V_\kappa 19$ genes. The map shows the integrated $V_\kappa 19A\text{-}J_2C_\kappa$ gene: exons (■), flanking regions and introns (▭), vector DNA (——), and DNA flanking the integration site (〰). Probe segments are shown below the map. H, *Hin*dIII; M, *Msp*I; X, *Xba*I; B, *Bam*HI. (c) Northern blot of nuclear (N) and cytoplasmic (C) poly(A)$^+$ RNA from 3-1(T) cells untreated (−) or exposed for 24 h to 10 μg of LPS per ml (+). The probes, defined in panel b and Fig. 1c, are nick-translated restriction fragments, except for U, which is a minus-strand riboprobe synthesized with T7 polymerase. Products of the exogenous κ^+ gene consist of a 9.5-kb transcript initiated upstream of the integration site and its derivatives (U) and a 4.8-kb transcript initiated at the normal $V_\kappa 19$ cap site and its 1.1-kb derivative (T). The products of the endogenous κ^0 gene consist of an 8-kb transcript and its ~1-kb derivative (κ^0). Kb, kb.

evident when nuclear and cytoplasmic poly(A)$^+$ RNA fractions from LPS-treated and untreated cells were compared. The products of the endogenous genes, revealed as coincident bands with the κ^0 and EC_κ probes (lanes 5 to 12), include the 8-kb nuclear transcript and a 1-kb processed derivative, which is mainly cytoplasmic. Two sets of κ^+ gene products were coincidentally revealed by the $V_\kappa 19$ and EC_κ probes (lanes 1 to 4 and 9 to 12, respectively). One set (T) includes the exclusively nuclear 4.8-kb κ^+ primary transcript and the mature 1.1-kb κ^+ mRNA, which is present in both nuclear and cytoplasmic RNA fractions. The other set (U) consists of a 9.5-kb nuclear transcript and its various processed derivatives. These components are revealed by an upstream probe, U (lane 13), as well as by the $V_\kappa 19$ and EC_κ probes. From the size of this transcript and its reactivity with the various probes, we judge that it is initiated about 2 kb upstream of the 5′ integration junction and extends to the C_κ poly(A) addition site. These data show that activation of the κ enhancer function by LPS treatment concurrently elicits transcription from the endogenous κ^0 promoter, the exogenous κ^+ promoter, and a cryptic promoter located upstream of the κ^+ integration site. The ability of the κ enhancer to drive transcription from two tandemly linked promoters (in this case, U and κ^+) is not unprecedented (2).

Sustained methylation of the transfected κ^+ gene in 3-1(T) cells. Southern blot analysis of the exogenous and endogenous κ genes in unstimulated 3-1(T) cells demonstrated that they are both fully methylated (Fig. 3b). This was evidenced by lack of cleavage at the *Hha*I sites in the J_κ region (three sites for the κ^0 gene and two sites for the κ^+ gene; Fig. 1c) and by *Hpa*II resistance at the *Hpa*II or *Msp*I site in the V region of the κ^+ gene (Fig. 2b). This effective de novo methylation of exogenous κ genes is not peculiar to the 3-1(T) transformant. Examination of these same restriction sites in LPS-responsive κ^+ genes introduced into the 70Z/3 pre-B cell line indicated that they were also heavily methylated and thus refractory to *Hha*I and *Hpa*II digestion (Fig. 3c). Furthermore, as was observed for the κ^0 genes in the parental 3-1 cells, the exogenous and endogenous κ genes in 3-1(T) cells remain heavily methylated after long-term exposure to LPS (about 14 generations in the experiment shown in Fig. 3b; at least 30 generations in other experiments). The persistent activity of the κ enhancers during such exposures was indicated by two observations. First, the poly(A)$^+$ RNA of the long-term-treated cells contained the short-lived nuclear transcripts (κ^0, κ^+, and U species), in addition to their more stable cytoplasmic derivatives (Fig. 3a). Second, all of these species were reduced to unstimulated levels within 2.3 generations after removal of the LPS (data not shown). These results demonstrate that an exogenous κ gene which is unmethylated at the time of transfection can, when introduced into the genome of a pre-B cell, acquire the characteristics of the endogenous κ genes, namely, sustained methylation during prolonged enhancer-dependent transcriptional activity. This implies that these characteristics do not have to be imprinted in the κ gene from previous developmental stages. Rather, they appear to be mainly determined by the particular combination of functions that affect this gene in the pre-B cell. These functions would presumably include the production of various *trans*-acting factors that interact with the κ enhancer, as well as structural components or activities that influence the methylation status of the κ genes.

The ability of transfected κ genes to display the characteristics of the endogenous κ genes may require integration into a relatively neutral site in which the structural properties of the introduced gene are not strongly influenced by neighboring chromosomal regions. In a preliminary experiment with a transfected κ^+ gene that was covalently linked to a selectable *Escherichia coli gpt* gene (18), we isolated a rare mycophenolic acid-resistant transformant in which the exogenous κ^+ gene was constitutively transcribed and hy-

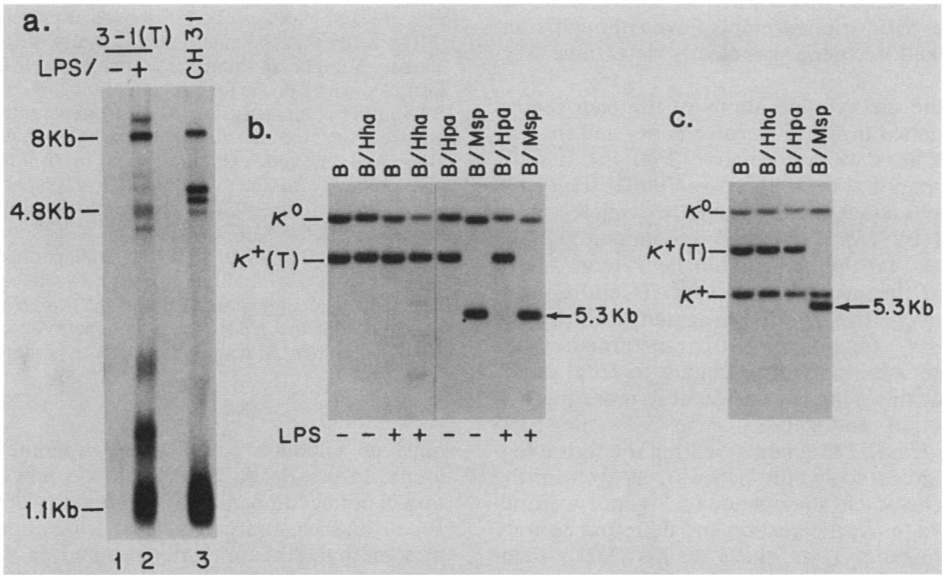

FIG. 3 Expression and methylation status of κ genes in untreated and long-term-LPS-treated pre-B-cell transformants. (a) Northern blot analysis of poly(A)$^+$ RNA from 3-1(T) cells untreated (−) or exposed for 14 generations to 10 μg of LPS per ml (+) (EC_κ probe). The sample of CH31 RNA (Fig. 1b) was included as a size marker and hybridization control. (b) Southern blot of DNA from the 3-1(T) cells used for panel a was digested with *Bam*HI (B) and either *Hha*I (B/Hha) or *Hpa*II (B/Hpa) or *Msp*I (B/Msp) and analyzed with the C_κ probe. Positions of the 12.5-kb fragment containing the endogenous κ^0 gene, the 8-kb fragment containing the exogenous κ^+ gene, and the 5.3-kb *Msp*I-*Bam*HI fragment from the κ^+ gene are indicated. (c) Similar Southern blot analysis of DNA from an LPS-inducible 70Z/3 transformant (Fig. 2a). Positions of the κ^0 and κ^+ endogenous genes and of the κ^+ exogenous gene [κ^+(T)] are indicated. Kb, kb.

pomethylated while the endogenous κ^0 genes remained completely LPS dependent and fully methylated. Since the *gpt* gene requires a relatively high level of expression to confer mycophenolic acid resistance, we may have inadvertently selected for an integration site with properties that can supplant or override the κ enhancer function.

Permissivity for κ enhancer function required for hypomethylation of transfected κ^+ genes in plasmacytoma cells. If the coupling between enhancer activity and hypomethylation were indeed developmentally regulated, one would predict that in mature B cells, transcriptionally active κ genes, exogenous as well as endogenous, should be hypomethylated. To test this proposition, we investigated the methylation status of transfected κ^+ genes that were stably integrated into the genomes of plasmacytoma cells. Moreover, the availability of plasmacytoma lines that differ markedly with respect to their permissivity for κ enhancer function (3) gave us an opportunity to examine the causal relationship between enhancer activity and hypomethylation in cells that are representative of mature B lymphocytes. One of these lines, S194, is typical of most plasmacytomas, in that it exhibits efficient transcription of exogenous κ genes. The nonpermissive line, S107, lacks the critical κ enhancer-binding protein NF-κB and therefore cannot express exogenous κ genes or other transfected genes that are under the control of the κ enhancer. Despite this deficiency, both of the endogenous κ genes in S107 cells (a κ^+ and a κ^- allele [15]) are expressed at normal levels (3).

Several stable transformants of S194 and S107 cells were obtained by protocols similar to those used for the pre-B-cell lines, namely, transfection by electroporation of a fragment containing the κ^+ gene ($V_\kappa 19A\text{-}J_2C_\kappa$ or a derivative, V21/19 [2]) either linked or unlinked to a fragment containing a selectable marker gene (pSV2*neo* or pSV2*gpt*), followed by selection for G418 or mycophenolic acid resistance. As detailed previously (2) and illustrated by the example in Fig. 4a, the S194 transformants exhibited substantial expression of the introduced κ^+ gene. In contrast, κ^+ expression was insignificant in the S107 transformants, even though transcription of the linked *neo* gene was clearly detectable (Fig. 4a).

An analysis of the methylation status of the endogenous and exogenous κ genes in these transformants and in their respective parental lines was informative (Fig. 4b). In S107 transformants and parental cells, the two *Hin*dIII fragments bearing the active endogenous κ^+ and κ^- alleles were efficiently cleaved by *Hha*I to yield a common 0.87-kb fragment, indicating that this *Hha*I site (the 3′-most site of those monitored in the previous analyses) is entirely unmethylated. However, the *Hin*dIII fragment bearing the inactive exogenous κ^+ gene of the S107 transformant (indicated by the arrow) was relatively resistant to *Hha*I digestion, indicating that this gene is considerably more methylated than the active endogenous genes. In the S194 transformants, the *Hin*dIII fragments bearing the four exogenous κ^+ genes (indicated by the arrows), as well as the fragment bearing the single endogenous κ^+ gene, were all completely sensitive to *Hha*I digestion and therefore entirely unmethylated at this site. Thus, these results clearly demonstrate that exogenous κ genes introduced into plasmacytoma cells do indeed remain unmethylated, provided that the cells are permissive for the κ enhancer function.

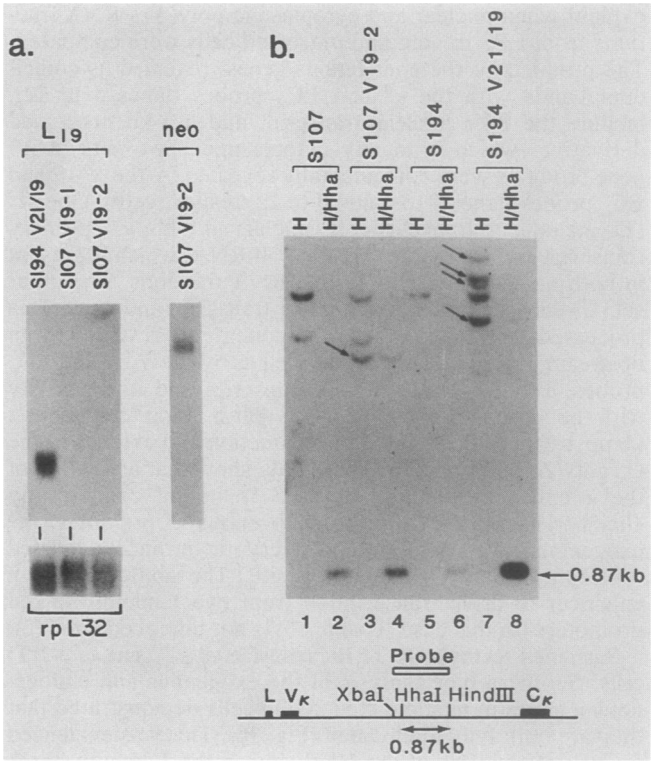

FIG. 4. Comparative expression and methylation status of exogenous κ^+ genes in S194 and S107 plasmacytoma cells. S194 cells were cotransfected with a derivative of a κ^+ gene (V21/19) and pSV2*gpt* and a series of stable transformant clones isolated as described previously (2). S107 cells were transfected with the κ^+ gene $V_\kappa 19J_2C_\kappa$ (Fig. 2) inserted at the *Bam*HI site of pSV2*neo*, and a series of stable G418-resistant transformants were isolated. (a) A Northern blot of cytoplasmic poly(A)$^+$ RNA from an S194 transformant and two independent S107 transformants were analyzed with a probe for the leader exon of the $V_\kappa 19$ gene (L_{19} [2]) and then rehybridized with a probe for ribosomal protein L32 mRNA (rpL32 [8]) to verify that the quality and quantity of RNA in each lane was similar. A duplicate sample from one of the S107 transformants was analyzed with a probe for a region of the linked pSV2*neo* gene (neo). (b) Southern blot analysis of DNA from parental S107 and S194 cells and from selected transformants S107 V19-2 and S194 V21/19-6. The DNA was digested with *Hin*dIII (H) or *Hin*dIII and *Hha*I (H/Hha) and probed with the *Xba*I-*Hin*dIII fragment shown below. The arrows indicate the *Hin*dIII fragments containing the exogenous κ^+ genes (one in the S107 transformant and four in the S194 transformant); the additional *Hin*dIII fragments contain the endogenous κ^+ genes (two in S107 and one in S194). Disappearance of the *Hin*dIII fragments and appearance of the 0.87-kb *Hha*I-*Hin*dIII fragment indicates that the *Hha*I site is not methylated; persistence of the *Hin*dIII fragment indicates that the site is methylated.

DISCUSSION

The results of our experiments (Table 1) indicate that there was a developmentally regulated change in the coupling between enhancer activity and hypomethylation of the κ locus. In pre-B cells, LPS-induced precocious κ transcription is not accompanied by hypomethylation, whereas at the B-cell and plasma cell stages, transcriptional activity and hypomethylation are tightly coupled. In these studies, hypomethylation serves as an indicator of structural differences in the chromatin domain that encompasses the interactions between the κ enhancer and promoter elements. Our experiments do not address the question of whether the methylation pattern is the primary determinant or a secondary consequence of these differences. That methylation patterns have the potential to regulate transcription via their effect on

TABLE 1. Transcriptional activity and methylation status of endogenous and exogenous κ genes in lymphoid cell lines representing different developmental stages

Developmental stage	Cell line[a]	NF-κB[b]	Transcriptional activity[c]		Methylation status[d]	
			Endogenous κ	Exogenous κ	Endogenous κ	Exogenous κ
Pre-B cell	3-1, 70Z/3					
	Without LPS	−	−	−	+	+
	With LPS	+	+	+	+	+
B cell	CH31		+		−	
Plasma cell	S194	+	+	+	−	−
	S107	−	+	−	−	+

[a] Although 70Z/3 cells contain a rearranged κ gene, they resemble pre-B cells in the characteristics studied here (20). The properties of the endogenous κ genes in CH31 and S194 cells have been observed in several other cell lines representing similar developmental stages (17, 20, 30).
[b] −, Absent; +, present.
[c] −, Inactive; +, active.
[d] −, Hypomethylated; +, methylated.

chromatin structure is evident from the results of several recently reported experiments (6, 13, 28, 35).

The relationship between structure and enhancer function is incorporated in a model for the developmental regulation of the κ locus (Fig. 5). In pre-B-cell lines, the endogenous κ^0 genes tend to be fully methylated and transcriptionally silent. These characteristics can also be acquired by initially unmethylated κ^+ genes that are transfected into the cells and

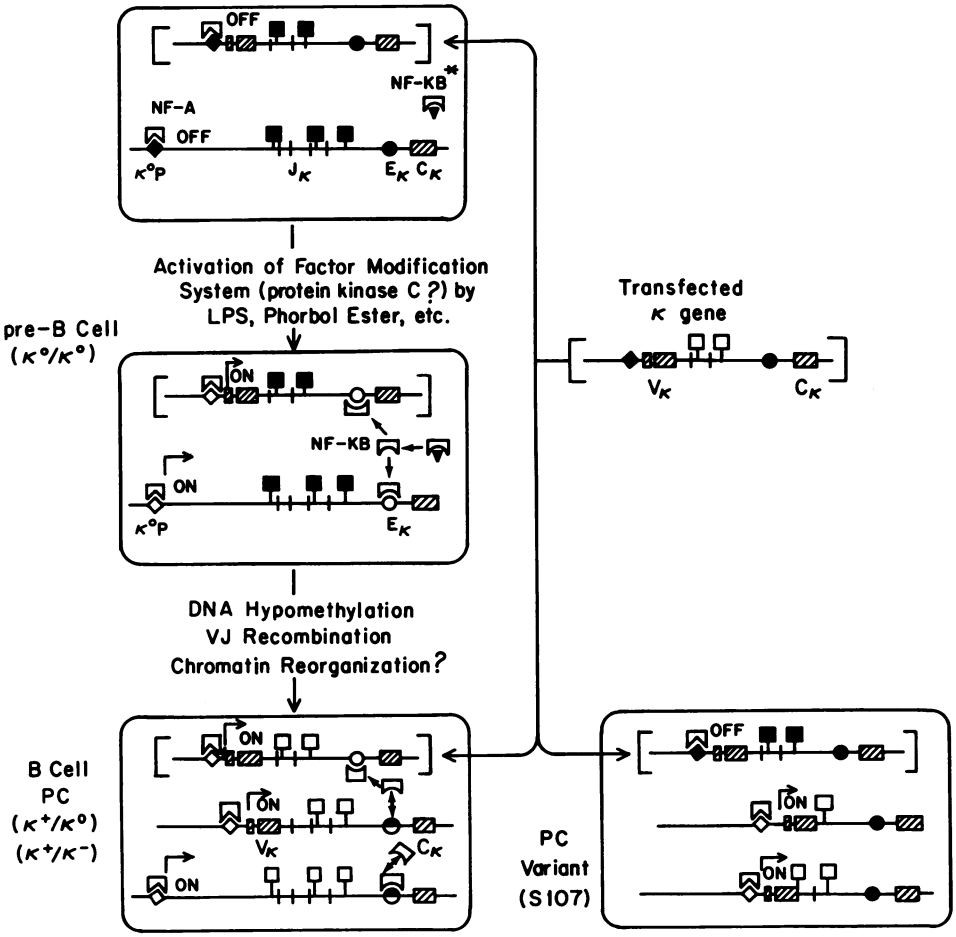

FIG. 5. Model for the developmental regulation of κ gene expression and the predicted fate of a transfected κ^+ gene. Symbols for the transfected κ^+ gene (in brackets) and the endogenous κ^0, κ^+, and κ^- alleles follow the conventions of the diagram in Fig. 1c. Inactive enhancer and promoter elements and methylated *Hha*I sites are designated by solid symbols; active elements and unmethylated sites are designated by open symbols. ▨, Exons.

integrated at neutral chromosomal loci. In these cells, the κ enhancer element is essentially nonfunctional because of the lack of a critical *trans*-acting factor NF-κB. The factors necessary for proper functioning of the κ promoters are apparently not limiting (3). NF-κB becomes available when these cells are treated with various agents, such as LPS and phorbol ester, which are believed to activate a posttranslational modification system, possibly protein kinase C, that converts an inactive form of the factor (NF-κB*) to a form with appropriate enhancer-binding properties (3, 27). Thus, transcription of both endogenous and exogenous κ genes is induced when the cells are exposed to LPS. These genes remain fully methylated, however, even after more than 20 successive cell doublings in a transcriptionally active state. Moreover, the function of the κ enhancer remains continuously dependent on NF-κB, as evidenced by the rapid cessation of transcription when LPS is withdrawn. Clearly, in these pre-B cells, enhancer-mediated transcriptional activity per se is not sufficient to cause hypomethylation of the κ genes.

In B-cell lymphomas, plasmacytomas, and hybridomas, the endogenous κ genes are generally hypomethylated and constitutively transcribed, irrespective of their rearrangement status (κ^+ or κ^- or κ^0). Furthermore, when exogenous κ^+ genes are transfected into such cells, they acquire these characteristics, provided that the cell is permissive for the κ enhancer function, which presumably establishes their transcriptional competence. In a variant plasmacytoma cell that lacks NF-κB, the transcriptionally active endogenous κ genes are entirely unmethylated, whereas an inert transfected κ^+ gene becomes largely methylated at the very same set of restriction sites. It appears, therefore, that the linkage of hypomethylation to enhancer-dependent activation of κ transcription develops during the transition from pre-B to B cells. This property, together with associated changes in the chromatin structure of the κ locus (25), may be sufficient to render the enhancer function dispensible or at least considerably less important for the maintenance of the transcriptionally active state. The fact that fully induced κ genes in pre-B cells and the constitutively expressed κ genes in more mature B cells are transcribed at roughly equivalent rates (11, 12, 19, 20) indicates that the methylated form of the gene, when driven by a properly functioning enhancer, can function as efficiently as the hypomethylated form.

In general terms, the above model implies that heritable alterations of methylation pattern and chromatin structure at a particular developmental stage can supersede the function of a regulatory element that was critical for expression at an earlier stage. A developmentally regulated change in the overall capacity for DNA and chromatin modification could result in selective alterations at particular gene loci, if the relative sensitivity to such modifications were influenced by local sequence differences or interactions with *trans*-acting factors. Such a change might concurrently affect the κ gene and the immunoglobulin heavy-chain (IgH) genes and thus engender a coordinate shift away from enhancer dependence in both immunoglobulin genes. Alternatively, the relevant change in IgH gene structure might precede the change in κ gene structure, in which case enhancer independence of IgH expression would occur at an earlier stage. From the available data on the methylation status of IgH genes in cell lines representing different developmental stages (5, 9, 30), it would appear that IgH hypomethylation is gradual, occurring first at the 5′ end of the gene in pre-B cells and later at the 3′ end of the gene in B cells. Which, if any, of these changes is related to the shift towards enhancer independence remains to be established.

Previous studies in which κ^+ genes linked to selectable marker genes were transfected into pre-B cells described transformants that constitutively express these κ^+ genes (23, 24), as well as an example that exhibits LPS-stimulated κ^+ expression (24). Since the organization and methylation status of these integrated κ^+ genes were not analyzed, it is difficult to relate these findings to our present results. We suspect, however, that at least some of the constitutively expressed κ^+ genes may have been hypomethylated, as was the case for the *gpt*-linked κ^+ gene in our rare pre-B-cell transformant. Conceivably, selection for the activity of linked marker genes might inadvertently also select for hypomethylated integration sites. Indeed, a significant level of κ^+ expression was occasionally observed with mutant genes that totally lacked the κ enhancer region (23). Another important consideration is the choice of host cells. Both studies used derivatives of the relatively unstable Abelson murine leukemia virus-transformed cell line 18-8. As has been noted previously (1), the descendants of this line display several characteristics e.g., tendency to give rise to κ^+-expressing subclones, predominant production of the secreted form of μ mRNA, J-chain expression, and ability to undergo an IgH class switch, which suggest that they have a propensity to mature beyond the pre-B-cell stage. Thus, the ability of these cells to support the constitutive expression of exogenous κ genes may be related to the fact that they are not really typical of lymphocytes frozen at the pre-B-cell stage. Still another factor that can influence the outcome of such experiments is the method of DNA-mediated gene transfer. For example, the calcium phosphate procedure frequently results in the combined integration of unlinked cotransfected genes at a single locus (22). With electroporation, which normally results in only a few integrated gene copies per cell (2), this possibility is remote.

Observations of the regulated expression of exogenous κ genes in Abelson murine leukemia virus transformants from transgenic mice (31) are generally consistent with the model discussed above. When the dosage of the κ transgene was not particularly high, the exogenous κ genes were transcriptionally silent in these pre-B cell lines. Since the developmental history of the κ transgenes parallels that of the endogenous κ genes, we would expect them to have a similar methylation pattern and chromatin structure if, as we have argued, these properties are determined by a combination of successive global changes and site-specific interactions. Although a rigorous verification of this conjecture awaits a more detailed structural analysis of the κ transgenes, the fact that a high proportion of these genes eventually become functional at the appropriate stage of development (32) suggests that it is likely to be valid.

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