A labile inhibitor blocks immunoglobulin κ -light-chain-gene transcription in a pre-B leukemic cell line

(*trans*-acting factor/cycloheximide/immunoglobulin gene)

RANDOLPH WALL*[†], MICHAEL BRISKIN*, CARLA CARTER*, HERMAN GOVAN*, ANNETTE TAYLOR[†],
AND PAUL KINCADE[‡]

*Molecular Biology Institute and [†]Department of Microbiology and Immunology, University of California, Los Angeles, School of Medicine, Los Angeles, CA 90024; and [‡]Oklahoma Medical Research Foundation, 825 N.E. 13th Street, Oklahoma City, OK 73104

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ABSTRACT The murine pre-B leukemic cell line 70Z/3 contains both an unrearranged immunoglobulin κ -light-chain gene and a functionally rearranged but silent κ -light-chain gene. Mitogenic stimulation of growing 70Z/3 cells with bacterial lipopolysaccharide (LPS) activates κ -light-chain-gene transcription and results in a 10- to 20-fold increase in cytoplasmic κ -light-chain mRNA. The induction of κ gene expression by LPS was probed by using an inhibitor of protein synthesis. Concomitant treatment of 70Z/3 cells with LPS and cycloheximide failed to block κ -light-chain mRNA accumulation, indicating that new protein synthesis is not required for the activation of κ -gene expression. Treatment of 70Z/3 cells with cycloheximide alone resulted in κ -mRNA induction equivalent to those produced by LPS alone. The κ mRNA synthesized in the presence of cycloheximide was intact and able to direct the synthesis of κ light chains. Nuclear transcription assays revealed that cycloheximide, like LPS, activated κ -gene transcription. These findings indicate that the *trans*-acting factors necessary for κ -light-chain-gene transcription are present in pre-B cells, but their activity is blocked by short-lived inhibitory proteins.

Immunoglobulin heavy- and light-chain genes are rearranged and expressed at different stages in B-cell development (reviewed in refs. 1 and 2). Pre-B cells contain rearranged μ genes and synthesize immunoglobulin μ heavy chains, but not light chains (reviewed in ref. 3). As pre-B cells develop into B cells, light-chain genes are rearranged and expressed and light chains are assembled into IgM. The murine pre-B leukemic cell line, 70Z/3, has been extremely useful in defining the molecular events in immunoglobulin κ -light-chain-gene activation (4). Unlike most pre-B cells, in which neither light-chain gene is rearranged, 70Z/3 cells contain an unrearranged κ -chain constant-region (C_{κ}) gene and a productively rearranged, but normally silent, variable-plus-constant-region ($V + C_{\kappa}$) gene (5, 6). Exposure of 70Z/3 cells to bacterial lipopolysaccharide (LPS) induces κ -gene transcription, leads to κ -mRNA accumulation, and results in a majority of the cells expressing surface IgM (4, 7-9). LPS induction of κ transcription in 70Z/3 results in appearance of DNase hypersensitivity at a region in both the rearranged and germ-line κ genes (6, 10) now shown by DNA transfection studies to be the κ -chain-gene enhancer element (11-14). The finding of altered chromatin configuration at the κ enhancer sequence as the first detectable consequence of LPS-induced κ transcription strongly suggests that the κ enhancer functions in the activation of specific gene transcription.

In analyzing the requirements for new macromolecular synthesis in LPS-induced κ gene transcription in 70Z/3, we

discovered that cycloheximide treatment failed to block κ induction by LPS. This finding led us to examine the effect of cycloheximide alone on κ -gene expression in 70Z/3 cells. Here we report that cycloheximide treatment of 70Z/3 cells induced κ mRNA increases as large as those produced by LPS. The κ mRNA produced during cycloheximide treatment was shown to be functional by its ability to direct the synthesis of κ light chains after removal of cycloheximide from treated cells.

Transcription assays in nuclei isolated from treated and control 70Z/3 cells showed that cycloheximide and LPS each activated transcription of κ genes without significantly altering transcription of μ genes. These findings indicate that κ gene expression in 70Z/3 pre-B cells is inhibited by one or more labile regulatory proteins that apparently block transcription.

MATERIALS AND METHODS

Cells. A clone of 70Z/3 cells (4), giving >90% κ -chain-positive cells after LPS induction, was used in these studies. Inductions with LPS or inhibitors were carried out on growing cells at 5×10^5 per ml in RPMI 1640 medium containing 5% fetal calf serum and 50 μ M 2-mercaptoethanol. Immunoprecipitations and analyses of immunoglobulin chains were carried out as described (15).

RNA Analysis. Total poly(A)⁺ RNA was prepared as described (16). RNA was glyoxal treated and analyzed by electrophoresis followed by blot-transfer hybridization (17). Cytoplasmic RNA slot blots were carried out using a Minifold II apparatus (Schleicher & Schuell) and the method of White and Bancroft (18). The probe DNA used for κ -chain RNA hybridization was a subcloned 2797-base-pair *Bgl* II genomic gene segment containing C_{κ} intron and coding sequences (19). The probe DNA used for μ -chain RNA hybridization was a subcloned 1208-base-pair *Hind* III gene segment containing $C_{\mu 3}$, $C_{\mu 4}$, μ_s , and 3' flanking sequences (20, 21).

Transcription Assays. Nuclei were prepared and nuclear transcription reactions were carried out as described by Manley *et al.* (22), except that incubations were for 20 min. RNA was purified (23) and hybridized to immobilized, denatured probe DNA in slots (2 μ g per slot) essentially as described by Wahl *et al.* (24), but without dextran sulfate. This concentration of DNA was determined to be in excess over the range of nuclear RNA hybridization inputs used. Hybridized filters were digested with RNase A (2 μ g/ml) and RNase T₁ (5 units/ml) in 0.3 M NaCl/0.03 M sodium citrate, pH 7, for 30 min at 25°C. Hybridization intensities were determined by densitometric scanning of autoradiograms from hybridized filters.

Abbreviation: LPS, bacterial lipopolysaccharide.

RESULTS

Induction of κ mRNA by Cycloheximide and LPS. Exposure of growing 70Z/3 cells to optimal concentrations of LPS produced 10- to 20-fold increases in cytoplasmic κ -light-chain mRNA in 12–14 hr (7, 8). In contrast to κ mRNA, the level of μ mRNA was not appreciably changed in LPS-treated 70Z/3 cells. To determine whether new protein synthesis is required for LPS induction, we treated 70Z/3 cells with LPS in the presence of cycloheximide (10 μ g/ml). RNA slot blot hybridizations (18) were used to analyze κ mRNA from treated 70Z/3 cells. A low level of κ mRNA was detected in untreated cells by the sensitive RNA slot blot assay (Fig. 1). This κ mRNA is produced by a small fraction of untreated 70Z/3 cells ($\leq 5\%$) that spontaneously express κ chains (4). LPS induction for 16 hr gave a 9-fold increase in κ mRNA over untreated control cells (Fig. 1). Treatment of 70Z/3 cells with both LPS and cycloheximide gave a 14-fold increase in κ mRNA level after 16 hr of treatment, indicating that new protein synthesis was not required for κ mRNA synthesis during LPS induction. Strikingly, cycloheximide treatment alone gave a 12-fold increase in κ mRNA. Thus, cycloheximide treatment produced an increase in κ mRNA comparable to that obtained with LPS.

The level of μ -heavy-chain mRNA increased slightly (1.3-fold) in the presence of LPS and decreased by a factor of 3–4 in cycloheximide in LPS plus cycloheximide. Both μ and κ mRNA from long-term cycloheximide-inhibited cells exhibited partial degradation, probably due to decreased cell viability, when analyzed by electrophoresis followed by blot hybridization (results not shown).

To minimize possible detrimental effects of long-term cycloheximide treatment on mRNA stability, we treated 70Z/3 cells with reduced levels of cycloheximide for 6 hr and compared the κ mRNA to κ mRNA from LPS-induced 70Z/3. As shown in Fig. 2, the κ mRNA in the cycloheximide-treated 70Z/3 cells was indistinguishable in size from that in LPS-treated 70Z/3 cells, confirming that inhibition of protein synthesis led to the accumulation of authentic κ mRNA. LPS and two concentrations of cycloheximide (0.2 and 2.0 μ g/ml) gave κ mRNA bands significantly increased over the κ mRNA band in untreated control 70Z/3 cells. Densitometric quantitation showed that LPS treatment for 22 hr gave a 20-fold increase in κ mRNA. Both cycloheximide doses gave approximately 6-fold increases in κ mRNA relative to untreated control 70Z/3 cells. Cycloheximide at 0.2 and 2.0 μ g/ml inhibited protein synthesis in 70Z/3 cells by 70% and 90%, respectively. At these cycloheximide doses the level of μ mRNA was not significantly affected in treated and control 70Z/3 cells (Fig. 2). Accordingly, differential effects on

FIG. 1. Induction of κ mRNA by cycloheximide (CHx) and LPS. Growing 70Z/3 cells were treated with LPS (10 μ g/ml), cycloheximide (10 μ g/ml), or cycloheximide and LPS (both at 10 μ g/ml) for 16 hr. Cytoplasmic RNA slot blots (17) were hybridized with ^{32} P-labeled nick-translated κ - or μ -specific probes (see *Materials and Methods*). Cycloheximide at 10 μ g/ml caused $>95\%$ inhibition of protein synthesis in 70Z/3 cells treated for 1 hr. Puromycin (50 μ g/ml) and emetine (10 μ g/ml) also induced κ mRNA in treated 70Z/3 cells (results not shown).

FIG. 2. Analysis of κ and μ mRNA from cycloheximide- and LPS-induced 70Z/3 cells. Total poly(A)⁺ RNA was glyoxal-treated and electrophoresed in 1.0% agarose gels (17). After transfer to nitrocellulose, the RNAs were hybridized with the μ -specific probe (A) or the κ -specific probe (B). Lanes: 1, untreated control; 2, LPS-induced (10 μ g/ml, 22 hr); 3, cycloheximide-treated (2.0 μ g/ml, 6 hr); 4, cycloheximide-treated (0.2 μ g/ml, 6 hr). μ_m and μ_s represent RNA species encoding the membrane-associated and the secreted forms of μ heavy chain. Cycloheximide at 2.0 and 0.2 μ g/ml inhibited protein synthesis in 70Z/3 cells 90% and 70%, respectively.

mRNA stability likely do not account for increases in κ mRNA in cycloheximide-treated cells.

Synthesis of κ Light Chain after Cycloheximide Treatment. We next determined whether the κ mRNA accumulated during cycloheximide inhibition of protein synthesis in 70Z/3 cells was able to direct the synthesis of κ light chains. Cells were exposed to cycloheximide for either 2 hr, followed by an 18-hr recovery without inhibitor, or 6 hr without a recovery period. A third sample of cells was incubated with LPS for 20 hr. Treated and control cells were washed and then incubated for 2 hr with [^{3} H]leucine in inducer- or inhibitor-free medium. Cell lysates from the treated samples and control cells were immunoprecipitated with rabbit anti-mouse κ -chain antiserum, and equal aliquots were compared by NaDodSO₄/PAGE (Fig. 3). Immunoprecipitated κ -chain bands were quantitated by densitometry. Cells treated with cycloheximide for 2 hr or 6 hr showed 2-fold or 4-fold increases, respectively, in κ light chains relative to the κ chains in untreated 70Z/3 cells. LPS stimulation for 20 hr gave a 15-fold increase in κ chains over the level in control cells. The κ light chains from cycloheximide-treated cells were of normal size and were apparently assembled with μ heavy chains into IgM, since both chains were precipitated by anti- κ antiserum. The multiple μ chains seen probably represent membrane (μ_m) and secreted (μ_s) molecules with varying degrees of posttranslational glycosylation (25, 26).

Cycloheximide Treatment Stimulates κ -Gene Transcription. We next tested the possibility that moderate doses of cycloheximide, like LPS (9, 27), stimulated κ mRNA accumulation by activating κ -gene transcription. We compared the transcription of κ and μ genes in nuclei isolated from LPS- and cycloheximide-treated cells and from untreated 70Z/3 cells. Labeled RNA from *in vitro* nuclear transcription assays (22–24) was hybridized to an experimentally determined excess of denatured μ or κ DNA in slot blots. Hybridized RNA was treated with RNase and quantitated by densitometry. *In vitro*-labeled RNA from nuclei of cells treated either with LPS for 6 hr or with cycloheximide for either 3 or 6 hr gave μ -specific hybridization approximately equivalent to that seen in the untreated sample (Fig. 4). Quantitation of μ -specific hybridization bands by densitometry showed that inhibition of protein synthesis for up to 6 hr reduced ongoing μ -gene transcription by 10–30%. In contrast, treatment of 70Z/3 cells with cycloheximide increased κ -gene transcrip-

FIG. 3. Synthesis of κ light chains after LPS or cycloheximide induction of 70Z/3 cells. Cells treated as described below were incubated for 2 hr with [^{3} H]leucine. Cell extracts were immunoprecipitated with affinity-purified κ -chain-specific antibodies, denatured, and electrophoresed in a 10% polyacrylamide/NaDodSO₄ gel (15). Cycloheximide-treated cells were washed to remove cycloheximide before labeling. Lane 1: untreated control. Lane 2: LPS-induced (10 μ g/ml) for 20 hr. Lane 3: cycloheximide-treated (0.2 μ g/ml) for 6 hr. Lane 4: cycloheximide-treated (0.2 μ g/ml) for 2 hr and then washed and incubated 18 hr in growth medium prior to labeling. Lane M: markers from IgM(κ)-producing W279 cells (15).

tion up to 5-fold over control nuclei (Table 1). The level of κ transcription in nuclei after 6 hr of treatment was reduced relative to 3 hr of cycloheximide treatment (Table 1), possibly reflecting an effect of longer inhibition of protein synthesis. LPS treatment for 6 hr increased κ transcription 4- to 6-fold over control nuclei. These findings indicate that both LPS and cycloheximide act directly through the activation of κ -gene transcription.

When the hybridization intensities of hybridized RNA from nuclear transcription reactions were normalized per kilobase of specific probe DNA, κ transcription in nuclei from cells treated with LPS for 6 hr was 30–40% of the value for μ transcription. In nuclei from cells treated with cycloheximide for 3 or 6 hr, normalized κ transcription ranged from 20 to 40% that of the μ gene. This implies that induction periods up to 6 hr result in only partial induction of κ transcription. This is consistent with the previous observation that approximately 12–14 hr (i.e., one cell generation for

FIG. 4. Effect of cycloheximide and LPS on κ - and μ -gene transcription. Nuclear transcription assays and the μ and κ hybridization probes are described in *Materials and Methods*. Labeled RNA from the transcription assays was hybridized to immobilized μ - and κ -specific probes (2 μ g per slot). Filters: 1, untreated control; 2, cycloheximide-treated (1.0 μ g/ml) for 3 hr; 3, cycloheximide-treated (1.0 μ g/ml) for 6 hr; 4, LPS-induced (10 μ g/ml).

Table 1. Stimulation of κ -light-chain-gene transcription by cycloheximide and LPS

Treatment	Fold induction	
	Exp. 1	Exp. 2
Cycloheximide (1 μ g/ml, 3 hr)	3.9	5.2
Cycloheximide (1 μ g/ml, 6 hr)	3.4	2.7
LPS (10 μ g/ml, 6 hr)	6.5	4.2

Nuclei were prepared from control or treated 70Z/3 cells. RNA isolated from *in vitro* nuclear transcription reactions was hybridized to immobilized κ -specific DNA (2 μ g of DNA per slot). RNase-resistant κ hybridization was quantitated by densitometric scanning of autoradiograms such as in Fig. 3. Fold induction represents the ratio of hybridization intensity values of experimental samples to untreated controls. Hybridization ratios for μ -heavy-chain-gene transcription in experimental samples to controls ranged from 0.7 to 1.4.

70Z/3 cells) was required for maximal κ induction by LPS (4, 8).

DISCUSSION

The novel aspect of the results reported here is the labile nature of the blocking factors that inhibit κ -light-chain-gene transcription in the 70Z/3 pre-B cells. Further, 70Z/3 cells appear to be fully competent for κ -gene transcription without synthesis of new proteins. Prolonged incubation with cycloheximide at high concentration (10 μ g/ml) resulted in partial breakdown of both κ and μ mRNA. However, these complications were not evident with lower doses and shorter durations of cycloheximide treatment in which authentic κ mRNA increased substantially without significant effect on the size or level of μ mRNA. Increases in cytoplasmic κ mRNA closely approximated increases in κ -gene transcription in nuclei isolated from cycloheximide- or LPS-treated 70Z/3 cells. Accordingly, we presume that cycloheximide, like LPS, stimulates κ -chain mRNA accumulation primarily through the transcriptional activation of silent κ -light-chain genes. It is interesting to speculate that other developmentally regulated genes in pre-B cells might be controlled similarly by negative regulatory factors.

A number of genes whose expression is either developmentally- or cell cycle-regulated have been found to be induced by cycloheximide and other inhibitors of protein synthesis. These include *c-myc* (28, 29), β - and γ -actin (30), β -interferon (31, 32), interleukin 2 (33), and adenovirus early-region (34, 35) genes. In these cases, cycloheximide stimulated ongoing gene expression during infection or after activation by a specific physiological inducer (i.e., superinduction effect). This has been interpreted to indicate that cycloheximide may be exerting its stimulatory effect by increasing the activity of genes that are already transcriptionally active through the inhibition of labile *trans*-acting proteins that control either the level or duration of transcription. The effect of cycloheximide on κ -gene expression in 70Z/3 cells differs from the effects in these systems in two important ways. First, cycloheximide treatment alone is apparently sufficient for activation of silent κ genes in 70Z/3 cells. Second, unlike these other systems, which show superinduction effects, cycloheximide and LPS together did not produce κ -mRNA levels significantly higher than those seen with either inhibitor or mitogen alone.

Increases in functional κ -mRNA levels and κ -gene transcription are readily seen within 2–3 hr of cycloheximide treatment. This suggests that the inhibitory factors repressing κ -gene transcription may be relatively short-lived proteins. Labile inhibitors could block transcription directly by binding the κ gene or by stabilizing the interaction of other negative regulatory factors with the κ gene. Alternatively, the inhibitors could prevent κ -gene transcription indirectly by binding

trans-acting positive regulatory factors, thereby preventing productive interactions with the κ gene. Although it is not possible to distinguish between these two alternatives at present, arguments below favor the first alternative.

The hierarchy of events in DNA rearrangements and alteration of chromatin structure in immunoglobulin gene activation and transcription are now being resolved (reviewed in ref. 2). Rearrangement of $V + C_{\kappa}$ gene segments in 70Z/3 cells does not result in κ -gene transcription despite the functional juxtaposition of all the control elements of a complete κ -light-chain transcription unit. However, the local chromatin structure at the κ -gene enhancer in uninduced 70Z/3 cells is subtly altered from that in nonlymphoid tissues such as liver or brain (10). This suggests that some alterations in chromatin conformation at the κ enhancer precede the appearance of DNase I-hypersensitivity, or transcriptional activation (10). The present results indicate that 70Z/3 pre-B cells contain the *trans*-acting factors necessary for κ transcription although these positive regulatory factors are unable to initiate transcription, possibly due to restrictive chromatin structure of the 70Z/3 κ -chain genes. The blocking factors indicated by these studies may alter the chromatin structure at the κ enhancer to prevent the association of factors involved in the formation of the activated, DNase-hypersensitive state.

These speculations are completely consistent with transfection experiments in which κ -light-chain genes introduced into Abelson-virus transformed pre-B cells were expressed in an enhancer-dependent manner (36, 37). The 18-81 pre-B cells used in these studies have both κ genes in the unrearranged configuration and presumably represent a pre-B cell stage earlier than 70Z/3. These transfection studies also indicate that pre-B cells contain *trans*-acting factors necessary for κ -light-chain-gene transcription. It is now evident that genes introduced into eukaryotic cells by transfection are more readily activated for transcription than their endogenous counterparts (38–41). This increased transcriptional activity is reflected by greater accessibility of transfected genes to DNase I cleavage (40). These studies suggest that the activities of positive, *trans*-acting regulatory factors play a secondary role to restrictive chromatin structure in gene activation and expression.

Recently, pre-B-cell lines, containing rearranged κ genes, have been established from κ -chain-gene transgenic mice (42). Expression of the rearranged κ genes was seen in pre-B-cell lines with high transgenic gene copy numbers but not in pre-B-cell lines with low copy numbers. This finding suggests that increased numbers of κ genes may override the inhibitory effects of blocking factors. It will be interesting to determine whether κ expression in the low copy number transgenic pre-B-cell lines is activated by inhibitors of protein synthesis.

Finally, the tissue-specific pattern of immunoglobulin gene expression has been generally assumed to reflect a lack of required *trans*-acting transcription factors in nonlymphoid cells (reviewed in ref. 2). However, a recent report showing cycloheximide activation of silent human heavy-chain genes transfected into mouse L cells (43) suggests that the restriction of immunoglobulin gene expression in some nonlymphoid cells also may result from labile inhibitory factors.

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