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Structures of Human Genes Coding for Cytokine LD78 and Their Expression

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LD78 is a member of a newly identified superfamily of small inducible proteins involved in inflammatory responses, wound healing, and tumorigenesis. Southern blot analysis of the *Eco*RI-digested human genomic DNAs, using previously isolated LD78 cDNA as a probe, showed that in each individual there are 4.2- and 4.8-kilobase-pair (kb) fragments and that some have an additional 6.5-kb fragment. The 4.2-kb fragment contained genomic DNA sequences corresponding to the LD78 cDNA and was named the LD78α gene. The 4.8-kb fragment contained similar sequences, showing 94% homology to the LD78α gene, and was named the LD78β gene. The LD78α gene was present in a single or a few copies per haploid genome, whereas the copy number of the LD78β gene and of the 6.5-kb fragment hybridizable to LD78 cDNA varied among the samples tested. Treatment of human myeloid cell lines HL-60 and U937 with phorbol 12-myristate 13-acetate (PMA) increased within 2 h cellular levels of the RNA hybridizable to LD78 cDNA. The human glioma cell line U105MG and primary culture of human fibroblasts also expressed the hybridizable RNA in response to PMA. Addition of cycloheximide had no apparent effect on this response in U937 cells and inhibited the response in fibroblasts, whereas it stimulated the response in HL-60 and U105MG cells. mRNA phenotyping experiments revealed that the LD78α and LD78β genes were both transcribed in PMA-stimulated U937 cells.

We formerly isolated a cDNA clone, named pLD78, from a cDNA library constructed on the poly(A)$^+$ RNA of human tonsillar lymphocytes stimulated with phorbol 12-myristate 13-acetate (PMA) and phytohemagglutinin, using a differential screening procedure (33). Sequence analysis of the pLD78 cDNA revealed that it codes for a protein of 92 amino acid residues, including a putative leader sequence. To obtain information on the function of this LD78 protein, we made a computer search of nucleotide and protein sequence data bases and found that it has significant similarity to small inducible mammalian proteins forming a new cytokine superfamily (for reviews, see references 4, 34, and 49). Although the exact functions of the members of this cytokine superfamily are unknown, a monocyte-derived neutrophil chemotactic factor, now called interleukin-8 (IL-8), is one of the best-characterized proteins (21).

Among the members of the superfamily, LD78 has 75% sequence similarity with murine macrophage inflammatory protein 1α (MIP-1α) (10), also called murine L2G25B (20). Accordingly, we presume that LD78 is a human counterpart of murine MIP-1α. Murine MIP-1α has already been demonstrated to have inflammatory and chemokinetic properties (11, 50). Recently, we found that high levels of LD78 gene transcripts are present in acute nonlymphotic as well as lymphotic leukemic cells; hence, LD78 is also probably involved in the neoplastic transformation of hematopoietic cells (51).

We have now obtained evidence for at least three different LD78-like genes in the human genome. Among them, we isolated and characterized two highly homologous LD78 genes. We also characterized the regulatory expression of LD78 mRNAs in human hematopoietic cells and nonhematopoietic cells. Analysis of the LD78 mRNAs by mRNA phenotyping experiments showed that both the LD78α and LD78β genes are transcribed in PMA-stimulated myeloid U937 cells.

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MATERIALS AND METHODS

Probes. The *Pst*I-*Ban*I fragment (0.7 kilobase pairs [kb]) prepared from pLD78 (33) was labeled by a random primer system (Takara, Kyoto, Japan) with [α-^{32}P]dCTP (3,000 Ci/mmol; 1 Ci = 37 GBq; Dupont, NEN Research Products, Boston, Mass.) and was used as an LD78 cDNA probe. The *Hin*dIII-*Xba*I fragment (0.5 kb) of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was prepared from pKS321 (42), and the *Xba*I-*Pvu*II fragment (0.6 kb) of human IL-2 cDNA was prepared from pHIG5-3 (23). These DNAs were labeled as described above and were used as controls for RNA and DNA blots. The IL-2 cDNA probe detects one 3.6-kb *Eco*RI fragment containing exons 3 and 4 of the IL-2 gene (16).

Preparation of genomic DNAs and Southern blot analysis. Human genomic DNAs were prepared from cultured cells and peripheral blood lymphocytes of healthy individuals as described previously (19). DNAs were digested with restriction enzymes, electrophoresed, blotted onto nitrocellulose filters (Schleicher & Schuell, Inc., Keene, N.H.), and hybridized with the LD78 cDNA probe in 50% formamide–5× SSC (SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 42°C, and the filters were washed at 56°C in 0.1× SSC–0.1% sodium dodecyl sulfate. After autoradiography at −80°C with an intensifying screen, the DNAs were rehybridized with the IL-2 cDNA probe. Relative copy numbers of the *Eco*RI fragments were estimated by scanning the fluorescent images on imaging plates, using a Bio-image analyzer (Fuji, Tokyo, Japan) (1).

Isolation of the human LD78α gene. Peripheral blood DNA was completely digested with *Eco*RI and fractionated by 10 to 40% sucrose density gradient centrifugation for 20 h. The fractions containing 4.2-kb fragments were collected, purified, ligated with λgt10 arms (Stratagene, La Jolla, Calif.), and packaged, using Gigapack Gold extracts (Stratagene).

Recombinant bacteriophages used to infect *Escherichia coli* NM514 and plated on agar plates. Two clones were isolated from about 3×10^5 plaques, using LD78 cDNA as a probe. For further analysis, one of the cloned fragments was subcloned into the *Eco*RI site of pUC18.

Sequence analysis. Plasmid DNAs containing the 4.2-kb *Eco*RI fragment and the previously cloned 4.8-kb *Eco*RI fragment of Lm LD-1 (33) were linearized by restriction enzyme digestions, and deletions were generated by digestion with BAL 31 (Takara). To form blunt ends, the digested ends were treated with DNA polymerase I large fragment (Takara). The resulting fragments of various lengths were excised and inserted into appropriate enzyme sites of pSP72 (Promega Biotec, Madison, Wis.). Sequencing reactions were done by the dideoxy method (38), using a modified T7 DNA polymerase (Sequenase; U.S. Biochemical Corp., Cleveland, Ohio), [α-^{35}S]dCTP (1,000 Ci/mmol; Dupont NEN), and T7 or SP6 promoter primers (Promega). Editing and analyses of the sequence data were done by GRASE and GENIAS programs (Mitsui Knowledge Industry, Tokyo, Japan).

Determination of the transcription initiation site. An oligonucleotide-5′-GGCAGCAGTGGAGACCTGCATGATT-3′, complementary to nucleotides 83 to 107 (according to the LD78α gene sequence; see Fig. 3A), was synthesized by using a Dupont Coder 300 DNA synthesizer and was purified by reverse-phase column (C18) chromatography (Hitachi, Tokyo, Japan). The oligonucleotide was labeled with T4 polynucleotide kinase (Takara) and [γ-^{32}P]ATP (6,000 Ci/mmol; Dupont NEN) and was used as a primer in primer extension analysis. Poly(A)$^+$ RNA was purified by using Oligotex-dT30 (Takara) from total cellular RNA prepared as described below. The primer was mixed with 3 μg of poly(A)$^+$ RNA or 10 μg of yeast tRNA in 20 μl of 75 mM KCl, heated to 90°C for 5 min, and cooled at room temperature for 20 min. Then 30 μl of a solution containing 5 mM each deoxynucleoside triphosphate (dNTP), 50 mM Tris (pH 8.3), 75 mM KCl, 10 mM dithiothreitol (DTT), 3 mM $MgCl_2$, and 200 U of Molony murine leukemia virus reverse transcriptase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was added, and the samples were incubated at 37°C for 1 h. The product was coelectrophoresed with sequencing reaction products prepared by extending the 4.2-kb *Eco*RI fragment from the same primer having no 5′-terminal phosphate. [α-^{32}P]dCTP (3,000 Ci/mmol) was used instead of [α-^{35}S]dCTP for the reaction.

Cell culture and stimulation. Human cell lines were provided by the following individuals: T. Hattori (Kumamoto University, Kumamoto, Japan), HL-60 (acute promyelocytic leukemia); N. Kurata (Fujita-Gakuen Health University, Aichi, Japan), U937 (histiocytic lymphoma); and J. Kuratsu (Kumamoto University), U105MG (glioma). All of these cells were grown in RPMI 1640 medium (GIBCO Laboratories, Grand Islands, N.Y.) supplemented with 10% fetal calf serum (Flow Laboratories, Inc., McLean, Va.), 50 U of penicillin per ml, and 50 μg of streptomycin per ml. Primary culture of human fibroblasts from the oral mucosa of a 4-month-old child and passaged four times was obtained from T. Kukita (Kyushu University, Fukuoka, Japan) and maintained in Dulbecco modified Eagle minimal essential medium (Flow Laboratories) supplemented with 10% fetal calf serum and antibiotics. The cells were treated with one or a combination of the following agents: PMA (Sigma Chemical Co., St. Louis, Mo.) at a final concentration of 10 ng/ml; cycloheximide (Sigma) at 10 μg/ml; bacterial lipopolysaccharide (LPS) (Difco Laboratories, Detroit, Mich.) at 10 μg/ml; and retinoic acid (Sigma) at 10 nM. U105MG cells and primary culture of human fibroblasts were treated with PMA at a final concentration of 50 ng/ml.

Preparation of cellular RNA and Northern (RNA) blot analysis. Total cellular RNAs were extracted by the guanidinium isothiocyanate method as described previously (7), electrophoresed in 1% formaldehyde-agarose gels (10 μg per lane), and blotted onto nitrocellulose filters. The filters were hybridized with LD78 cDNA probe in 50% formamide–5× SSC at 42°C and were washed at 65°C for 1 h in 0.1× SSC–0.1% sodium dodecyl sulfate. After autoradiography, the hybridized probe was removed by placing the filters in boiling water for 40 min; then the same filters were rehybridized with the GAPDH cDNA probe. mRNAs levels were estimated as described above for Southern blot analysis.

mRNA phenotyping. We used two methods to separately estimate the levels of messages transcribed from two closely related LD78 genes. For the first method, we used the polymerase chain reaction (PCR) as follows. Complementary DNA synthesis and subsequent amplification of the cDNAs by PCR was performed in a single tube according to Doherty et al. (12) as follows. Approximately 0.5 μg of poly(A)$^+$ RNA was mixed, in a total volume of 20 μl, with a solution containing 1.6 μg of oligo(dT)$_{12-18}$ (Pharmacia, Uppsala, Sweden) 1 mM each dNTP, 80 U of RNasin (Promega), 50 mM Tris (pH 8.3), 75 mM KCl, 10 mM DTT, 3 mM $MgCl_2$, and 200 U of Molony murine leukemia virus reverse transcriptase. After incubation at 37°C for 30 min, the cDNA was diluted into 200 μl of a solution containing 0.2 mM each dNTP, 35 mM Tris (pH 8.3), 50 mM KCl, 7 mM DTT, 2 mM $MgCl_2$, and 50 pmol of two oligonucleotide primers. The mixture was heated at 90°C for 90 s, and then 6.5 U of *Taq* DNA polymerase (Takara) was added. DNA amplification was performed on an ASTEC Program temperature control system PC-500 (Fukuoka, Japan) for 35 cycles: 1 min at 55°C for annealing, 2 min at 72°C for primer extension, and 1 min at 94°C for denaturation. Two kinds of primers were prepared for PCR. One has a 5′-TTCTTGGC TCTGCTGACACTCGAGC-3′ sequence and was used for the synthesis of noncoding strands; the other has a 5′-CCGA TCACAGCCCTGAACAAAAGCA-3′ sequence and was used for the synthesis of coding strands. The locations of these sequences are shown in Fig. 3A. Amplified DNAs were digested with *Taq*I or *Bst*NI (Takara) and were electrophoresed on a 4% NuSieve GTG agarose gel containing ethidium bromide.

For the second method, we used primer extension. A primer 5′-GCGTGTCAGCAGCAAGTG-3′, complementary to nucleotides 157 to 159 and 848 to 862 (according to the LD78α gene sequence; see Fig. 3A), was synthesized and 5′ labeled, and primer extension was performed as described above. Products were analyzed on a 6% denaturing polyacrylamide gel. Sequencing reaction products of M13mp18 DNA obtained by using a standard primer were coelectrophoresed and used as size markers.

Propagation of *E. coli* cells carrying recombinant DNAs was performed in accordance with the guidelines for recombinant DNA research issued by the Ministry of Education, Science and Culture of Japan.

RESULTS

Southern blot analysis of human genomic DNAs. We had already isolated two phage clones carrying the 4.8-kb *Eco*RI fragment hybridizable to LD78 cDNA from a gene library constructed on human placental DNA. Partial nucleotide

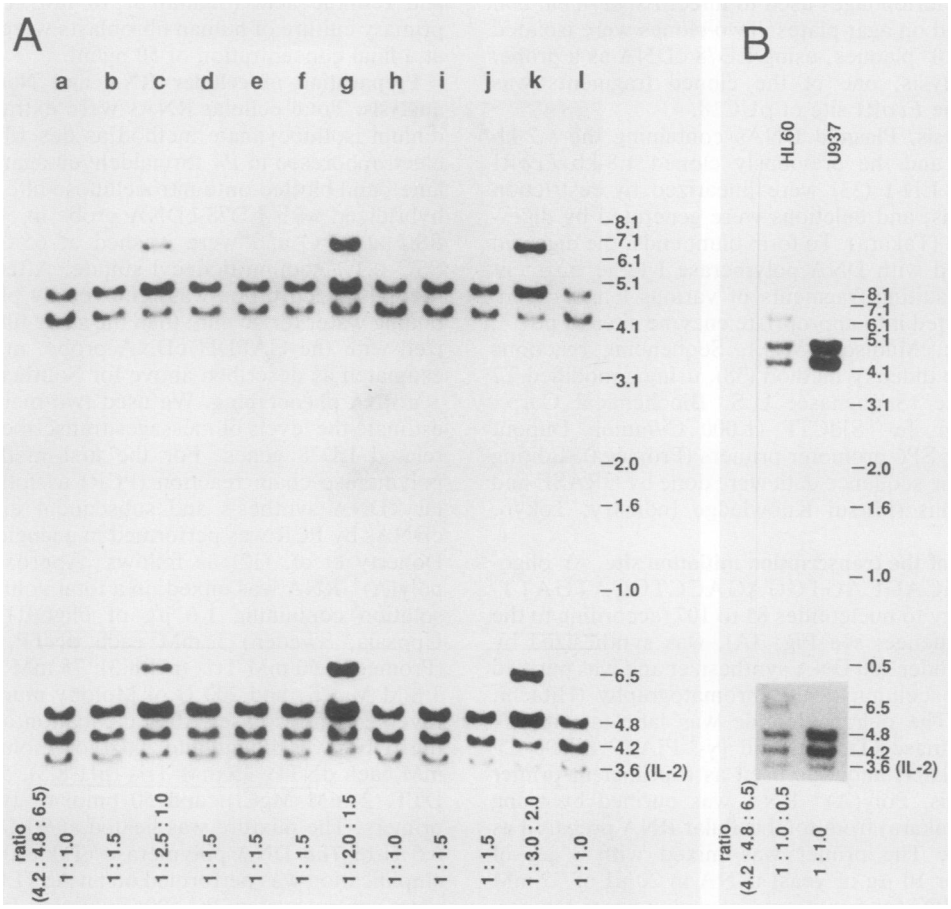


FIG. 1. Southern blot analysis of human genomic DNAs. DNAs (10 μg each) were digested with *Eco*RI, fractionated on 0.6% (A) or 0.8% (B) agarose gels, transferred onto nitrocellulose filters, and hybridized with the LD78 cDNA probe. (A) DNAs isolated from human peripheral blood lymphocytes of 12 individuals; (B) DNAs isolated from cultured HL-60 and U937 human cells. After autoradiography, the filters were rehybridized with the IL-2 cDNA probe. The autoradiograms are shown in the middle. The relative copy numbers of the fragments in each digest were calculated by using a Fuji Bio-image analyzer (1) and are shown at the bottom. Sizes (in kilobase pairs) of markers (1-kb DNA ladder size marker; Bethesda Research Laboratories) and fragments are shown to the right of each panel.

sequence analysis of this fragment showed that the sequence of the first exon was highly homologous but not identical with that of LD78 cDNA (33). To examine the possibility that there is another LD78 gene having exon sequences identical with those of LD78 cDNA, we isolated human genomic DNAs from peripheral blood of 12 healthy individuals and from two cultured human cell lines, HL-60 and U937. These genomic DNAs were digested with *Eco*RI, Southern blotted, and hybridized with the LD78 cDNA probe (Fig. 1). There were two bands of 4.2 and 4.8 kb in all of these DNAs and an additional band of 6.5 kb in 4 of 14 samples.

To correct for variations in the quantities of genomic DNAs applied per lane, the filters were rehybridized with a human IL-2 cDNA (23) probe without removing the prehybridized LD78 probe. The IL-2 gene is present in a single copy per haploid genome in every individual (16, 23) and was used as an internal control. After these filters were placed to contact an imaging plate for 1 h, we confirmed from fluorographic images that the intensity of the 4.2-kb fragment was proportional to that of the IL-2 fragment in each sample, i.e., an approximately 2:1 ratio (see middle panels of Fig 1A and B). In this experiment, we used the LD78 and IL-2 probes having approximately the same specific activities. Accordingly, this result indicates not only that the copy number of the 4.2-kb fragment is constant in each individual but also that it is probably present in a single or a few copies per haploid genome. Further experiments will be needed to determine the exact copy number of the fragment. In contrast, the copy numbers of the 4.8- and 6.5-kb fragments apparently varied among individuals (Fig. 1). When we assume that the 4.2-kb fragment is present in a single copy, the copy number of the 4.8-kb fragment in each individual is estimated to be 1, 1.5, 2, 2.5, or 3, and that of the 6.5-kb fragment is estimated to be 0, 1, 1.5, or 2 (lower panels of Fig. 1A and B). These results suggest that the copy numbers of the 4.8- and 6.5-kb fragments in the allelic chromosomes are not necessarily identical.

Structures of the LD78 genes present in the 4.2- and 4.8-kb *Eco*RI fragments. To characterize the structure of the gene present in the 4.2-kb *Eco*RI fragment, we cloned this fragment from a λgt10 library constructed on size-fractionated genomic DNAs (see Materials and Methods). By restriction mapping, we found that these two independently isolated clones contain the same genomic DNA insert. We compared a restriction map of the 4.2-kb fragment with that of the previously cloned 4.8-kb fragment and found that they are similar over a region of more than 3.5 kb (Fig. 2). Nucleotide

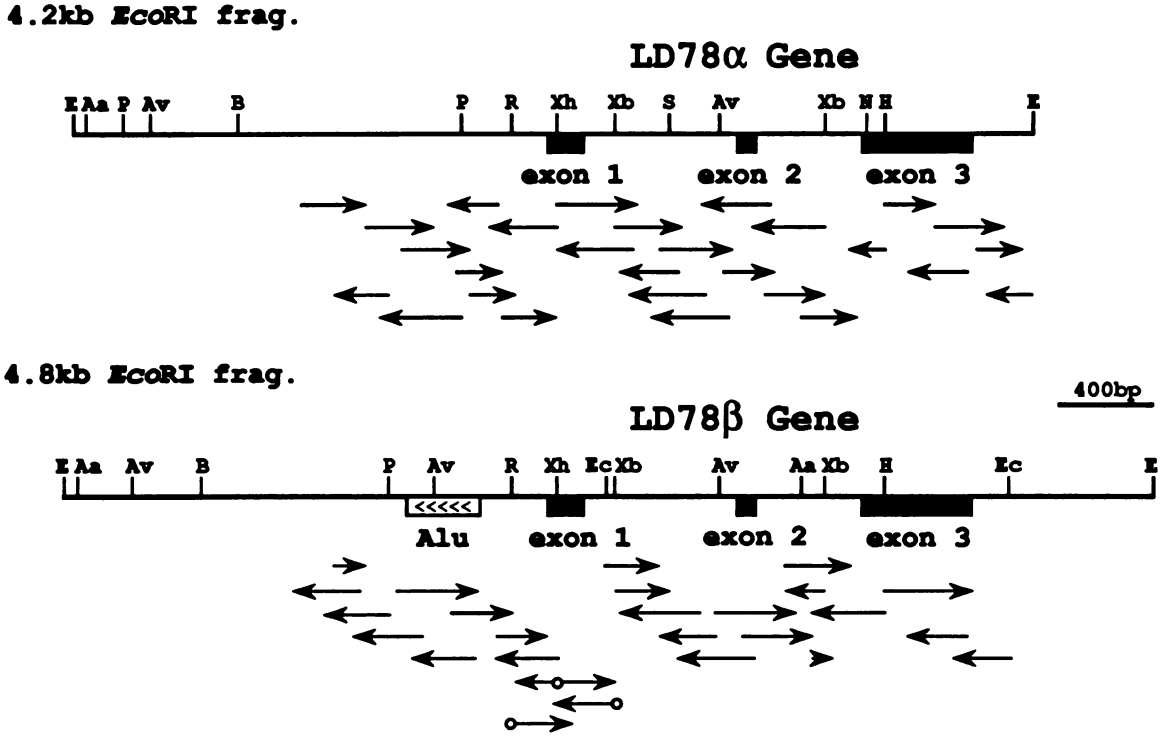


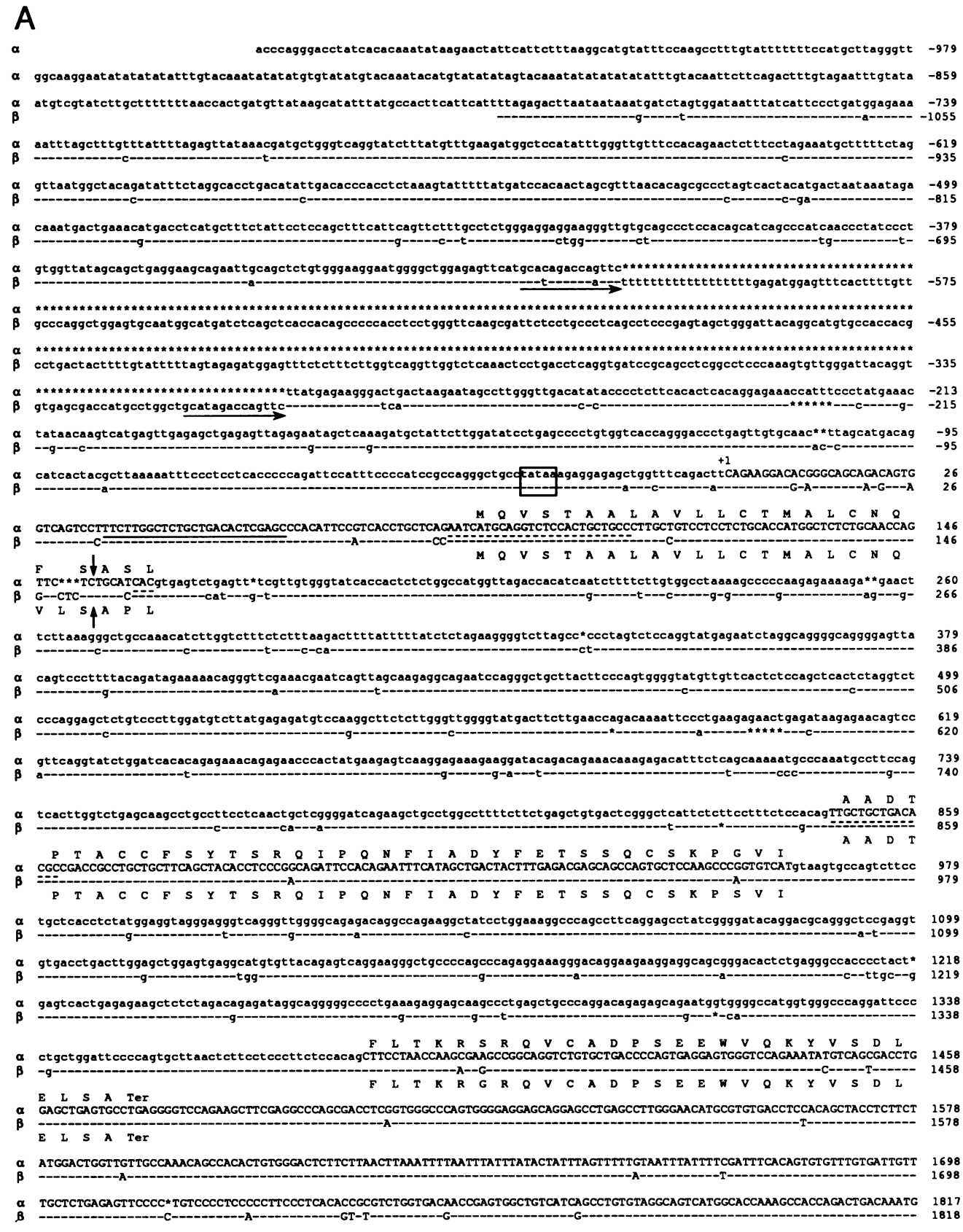
FIG. 2. Restriction maps of LD78 genes. The 4.2-kb *Eco*RI fragment is an insert present in phage clone Lm LD-3 isolated from a size-fractionated λgt10 genomic library. Isolation of the 4.8-kb fragment contained in a Charon 4A phage clone, Lm LD-1, was reported previously (33). Arrows beneath the maps represent the sequencing strategy. Sequencing reactions already performed (33) are shown by arrows with open circles. Solid boxes denote exons. The position and orientation of the *Alu* sequence in the 5′-flanking region of the LD78β gene are also indicated. Restriction sites: *Aat*I (Aa), *Ava*I (Av), *Bam*HI (B), *Eco*RI (E), *Eco*RV (R), *Eco*47III (Ec), *Hin*dIII (H), *Nae*I (N), *Pvu*II (P), *Sac*I (S), *Xba*I (Xb), and *Xho*I (Xh).

sequences of the genes in these two fragments (Fig. 3A) were determined by the strategy illustrated in Fig. 2. Each fragment contained one complete gene, and both genes were composed of three exons interrupted by two introns located at the same positions. The gene present in the 4.2-kb fragment was named the LD78α gene; the sequences of its exon regions were identical with those of the LD78 cDNA. Since we found that there are five mismatches between the sequence of the LD78α gene and the previously reported LD78 cDNA sequence (33), we resequenced the LD78 cDNA and corrected its sequence as follows: A at nucleotide position 264 and C at 678 were changed to G and T, respectively; and C at nucleotide position 319, A at 353, and C at 676 were added (33). This corrected LD78 cDNA sequence proved to be identical with that of exons of the LD78α gene. These corrections produce no change in the LD78 amino acid sequence.

The other gene, present in the 4.8-kb fragment, showed as high as 94% homology to the LD78α gene and was named the LD78β gene (Fig. 3A). Since the 6.5-kb *Eco*RI fragment was also hybridizable to the LD78 cDNA, it probably contains a third LD78-like gene. Although we have not cloned the LD78-like gene in this fragment, results of our preliminary genomic Southern blot analysis using several restriction enzymes showed that it differs from the LD78α and LD78β genes (data not shown); therefore, we will hereafter refer to the gene as LD78γ.

To determine the transcription initiation site precisely, primer extension was carried out by using poly(A)$^+$ RNA from U937 cells stimulated with PMA and LPS for 5 h (see below). Since we used a 5′-labeled primer complementary to the 5′ noncoding regions of both the LD78α and -β mRNAs (Fig. 3A), the reverse transcripts of both mRNAs were expected to have the same lengths. The elongated products were sized on a 6% denaturing polyacrylamide gel with a sequence ladder prepared by using the LD78α gene as a template and extension from the same primer having no 5′-terminal phosphate (Fig. 4). One major band and one minor band, which is three nucleotides shorter than the major band, were observed, but both bands were approximately one-half nucleotide out of phase. Since the elongated fragment containing a 5′-terminal phosphate migrates one-half nucleotide faster than the corresponding fragment generated by the sequencing reaction using the same primer having no 5′-terminal phosphate (39), the positions of both bands have to be retarded by one-half nucleotide to compensate for the difference in migration. Thus, the 3′-terminal nucleotide of the major band was mapped to the cytidine residue at +1 (Fig. 3A). This initiation site is preceded by the typical TATA box, located at −28 in both genes. The 3′-terminal nucleotide of the minor band corresponds to the 5′-terminal nucleotide of the LD78 cDNA, and we do not know at present whether the band represents a partial elongation product or a minor transcription initiation product. These results also indicate that the LD78α gene and probably the LD78β gene are transcribed from the same nucleotide position(s).

The LD78β gene has no base substitution(s) either in the coding regions to generate a stop codon(s) or in the exon-intron boundary sequences. Moreover, percent homology of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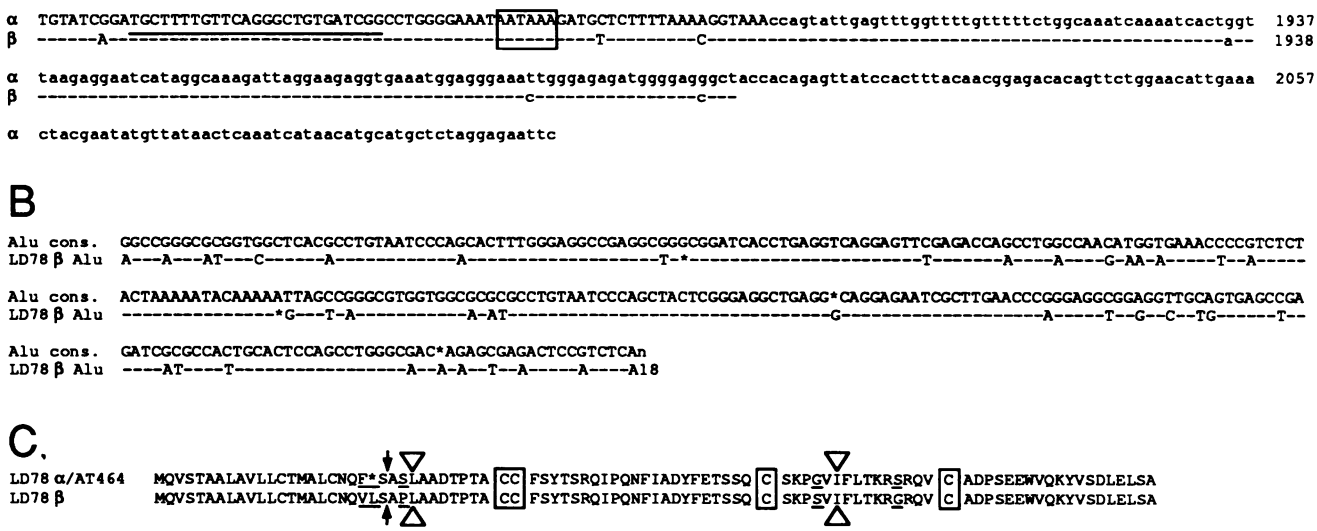

FIG. 3. Nucleotide sequences of the LD78α and LD78β genes. The nucleotide sequence data reported here will appear in the DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under the accession numbers D90144 (LD78α gene) and D90145 (LD78β gene). (A) Comparison of the nucleotide sequences of the LD78α and LD78β genes. Exon sequences are shown by uppercase letters. In the sequence of the LD78β gene, the nucleotides identical with those of the LD78α gene are shown by dashes. The major transcription initiation site is numbered +1 (Fig. 4). Vertical arrows indicate the putative cleavage sites of the leader sequences. The TATA box and the polyadenylation signal sequence AATAAA are boxed. Direct repeats flanking an *Alu* element in the 5′-flanking region of the LD78β gene are indicated by arrows. Nucleotide sequences of the primers used for PCR (Fig. 6) are marked by solid underlines. Sequences complementary to primers used for primer extension analyses (Fig. 4 and 7) are marked by dashed underlines. The one-letter amino acid code indicates the open reading frames. Ter, Termination codon. (B) Comparison of the *Alu* sequence in the 5′-flanking region of LD78β gene with the *Alu* consensus sequence. Nucleotides identical with the *Alu* consensus sequence (17) are shown by dashes. Asterisks are inserted to maximize the homology. (C) Alignment of amino acid sequences of LD78α (AT464) and LD78β. Amino acid differences between LD78α and LD78β are shown by underlines. The four cysteine residues conserved in the new cytokine superfamily members are boxed. Putative cleavage sites of leader sequences and positions of introns are indicated by arrows and triangles, respectively. An asterisk is inserted to maximize the homology.

the coding regions between the LD78α and LD78β genes was 96%, whereas the percent homology of the noncoding regions including introns was 93%. These results suggest that the LD78β gene is transcriptionally active. The 5′-flanking region of the LD78β gene, spanning from nucleotides −614 to −313, contains one *Alu* sequence flanked with incomplete direct repeats of 14 base pairs (bp), probably generated by target site duplication (48) (Fig. 3A and B). This *Alu* sequence was located in the opposite orientation relative to the direction of transcription of the LD78β gene.

Amino acid sequences of the proteins deduced from the nucleotide sequences of the LD78α and LD78β genes are shown in Fig. 3C. The cleavage sites for LD78α and LD78β leader peptides were predicted from their homologies to the processing sites of other superfamily proteins as well as from the rule of von Heijne (47). One contiguous three-base insertion and four nucleotide substitutions in the coding region of the LD78β gene caused one amino acid insertion and one amino acid substitution in the leader peptide and three amino acid substitutions in the mature protein (Fig. 3C). The four cysteine residues conserved among the superfamily members (34) were also retained in the LD78β protein.

Regulation of LD78 gene expressions. We next examined the regulation of expression of the LD78 genes in the human myeloid cell lines HL-60 and U937 (Fig. 5A). In response to treatment with PMA, both cells differentiate into monocyte-macrophage-like cells (8, 24). We stimulated these two cell lines for 5 h with various combinations of agents, including PMA, LPS, cycloheximide, and retinoic acid, extracted total cellular RNAs, and analyzed them by Northern blotting for sizes and amounts of LD78 mRNAs. Under the stringency used in this experiment, we expect that the LD78β mRNA, if present, would also be detectable using by the LD78 cDNA probe because it has high sequence similarity with LD78α mRNAs. To normalize the amounts of RNAs examined in each experiment, we rehybridized the filters with a human GAPDH cDNA probe.

Whereas there were no detectable LD78 mRNAs in nonstimulated HL-60 and U937 cells, both cells showed increased levels of the LD78 mRNAs after the stimulation with PMA (Fig. 5A). The sizes of these induced LD78 mRNAs were approximately 0.9 kb and identical to that of the LD78 mRNA originally detected in human tonsillar lymphocyte cells (33). The addition of cycloheximide, a protein synthesis inhibitor, to HL-60 cells stimulated with PMA increased the level of LD78 mRNAs fourfold in comparison with the level observed after the PMA stimulation. Whereas HL-60 cells are induced to differentiate into granulocytes in response to retinoic acid treatment (3), they were not induced to accumulate LD78 mRNA with this treatment (Fig. 5A).

We found that treatment of U937 cells with LPS or cycloheximide had little effect on the level of LD78 mRNA (Fig. 5A). On the other hand, treatment of U937 cells with a combination of PMA and LPS slightly increased the level of LD78 mRNAs in comparison with the level observed after stimulation with PMA alone, and the addition of cycloheximide did not inhibit the induction of LD78 mRNA by PMA and LPS (Fig. 5A).

We then examined expression of the LD78 genes in nonhematopoietic cells, such as primary culture of human fibroblasts and the human glioma cell line U105MG (2) (Fig. 5A). Stimulation of these cells with PMA resulted in increases in the cellular level of LD78 mRNA (Fig. 5A). The addition of cycloheximide to the primary culture of fibroblasts prevented this induction, but it did increase the level

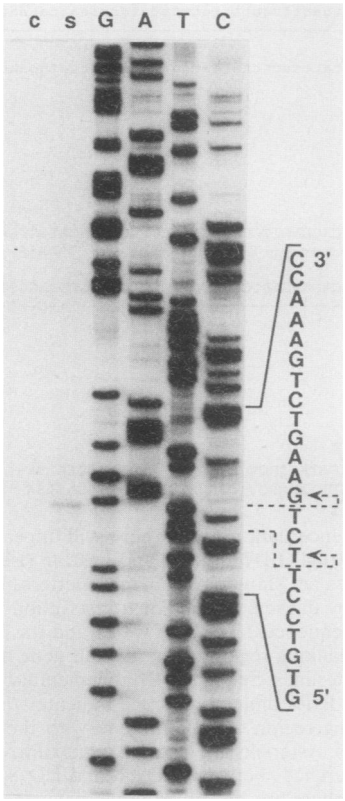

FIG. 4. Primer extension analysis. A 5'-labeled synthetic oligonucleotide complementary to nucleotides 83 to 107 (according to the LD78α gene sequence; Fig. 3A) was hybridized to 3 μg of poly(A)$^+$ RNA from U937 cells stimulated with PMA and LPS for 5 h. The elongation was carried out as described in Materials and Methods, and the products were fractionated on a 6% denaturing polyacrylamide gel (lane s). As a control, 10 μg of tRNA was used instead of poly(A)$^+$ RNA (lane c). To locate the transcription initiation site precisely, sequencing reaction products prepared by using the 4.2-kb *Eco*RI fragment as a template and by extension from the same primer having no 5'-terminal phosphate were coelectrophoresed. The nucleotide sequence on the right side is for the coding strand.

of LD78 mRNA 2.5-fold in U105MG cells. We also found that another human glioma cell line, U251MG, and human bladder carcinoma cell line U5637 did not induce LD78 mRNAs in response to stimulation with serum or PMA (data not shown).

We examined the kinetics of LD78 mRNA induction in HL-60 and U937 cells. Total cellular RNAs were extracted at different times after stimulation and analyzed for the induction of LD78 mRNAs. LD78 mRNAs were detected within 2 h after either the combined stimulation of HL-60 cells with PMA and cycloheximide or the combined stimulation of U937 cells with PMA and LPS (Fig. 5B). LD78 mRNAs in these cells reached maximal levels after 4 to 8 h of stimulation and then gradually decreased. However, LD78 mRNAs were clearly detectable even after 24 h of the stimulation.

Detection of LD78α and LD78β mRNAs in HL-60 and U937 cells. To examine the regulatory mechanisms of LD78 gene expression, we separately estimated the levels of LD78α and LD78β mRNAs by two methods. In the first method, we applied the recently described mRNA phenotyping technique (35) and prepared poly(A)$^+$ RNAs from HL-60 cells stimulated for 5 h with the combination of PMA and cycloheximide. We also prepared poly(A)$^+$ RNAs from U937 cells stimulated for 5 h with the combination of PMA and LPS (Fig. 5A). By using these RNAs as templates, cDNAs were first synthesized with oligo(dT) as a primer, and then the synthesized cDNAs were amplified with *Taq* polymerase in the presence of two specific primers hybridizing to the two separate sequences common to both types of mRNAs (Fig. 3A). The lengths of the amplified cDNA fragments were estimated to be 708 bp for the LD78α cDNA and 711 bp for the LD78β cDNA. These fragments were digested with *Taq*I or *Bst*NI because LD78α cDNA contains one additional *Taq*I site and LD78β cDNA contains one additional *Bst*NI site. The predicted restriction fragments are shown in Fig. 6B.

The LD78α cDNA-specific fragments marked with circles in Fig. 6B are two *Taq*I fragments of 183 and 179 bp and one 314-bp *Bst*NI fragment. All of these fragments were detected in amplified cDNAs prepared on the poly(A)$^+$ RNAs extracted from the stimulated HL-60 and U937 cells (Fig. 6A). The LD78β cDNA-specific fragments marked with triangles are one 362-bp *Taq*I fragment and two *Bst*NI fragments of 110 and 207 bp. All of these fragments were apparent in digests derived from the stimulated U937 sample, although the intensities were significantly weaker than those of the LD78α cDNA-specific bands (Fig. 6A). In contrast, the LD78β cDNA-specific bands were hardly visible in the amplified cDNAs prepared on the poly(A)$^+$ RNAs extracted from stimulated HL-60 cells as well as from nonstimulated U937 cells.

Although we could not detect LD78 mRNAs in the RNAs prepared from nonstimulated HL-60 and U937 cells even after the prolonged exposure of the Northern blots (Fig. 5A and data not shown), the LD78α cDNA-specific bands were clearly visible in the amplified cDNAs prepared on the poly(A)$^+$ RNAs extracted from the nonstimulated HL-60 and U937 cells (Fig. 6A). We speculate that this result is due to the high sensitivity of the PCR method and that the intensities of the LD78α cDNA-specific bands derived from the stimulated samples do not necessarily reflect the cellular level of LD78α mRNA because of the possible shortage of primers during the late cycles of the amplification reaction. In addition to these predicted bands, one faint, fast-migrating band was observed in the stimulated samples (Fig. 6A). We do not know whether this fragment is derived from a transcript related to the LD78 mRNA or whether it is an artifact. The LD78β gene seemed to be transcribed in the stimulated U937 cells, and the level of LD78β gene transcript was lower than that of the LD78α gene transcript.

In the second method used for mRNA phenotyping, we took advantage of a three-base deletion present in the first exon of the LD78α gene but not in the corresponding region of the LD78β gene (Fig. 3A). A synthetic oligonucleotide complementary to the sequence present immediately downstream from the deletion point (Fig. 3A) was 5' labeled and used as a primer in this experiment. From the result shown in Fig. 4, the elongation products prepared from this primer by reverse transcriptase were expected to contain three kinds of reverse transcripts with different lengths (Fig. 7B). The elongated LD78α cDNA-specific fragments would be a mixture of one major and one minor transcript of 174 and 171 nucleotides in length, respectively; similarly, the LD78β cDNA-specific fragments would be a mixture of fragments 177 and 174 nucleotides in length. Figure 7A shows the result obtained by using the poly(A)$^+$ RNA from the stimulated U937 cells as a template. Three bands of the expected sizes

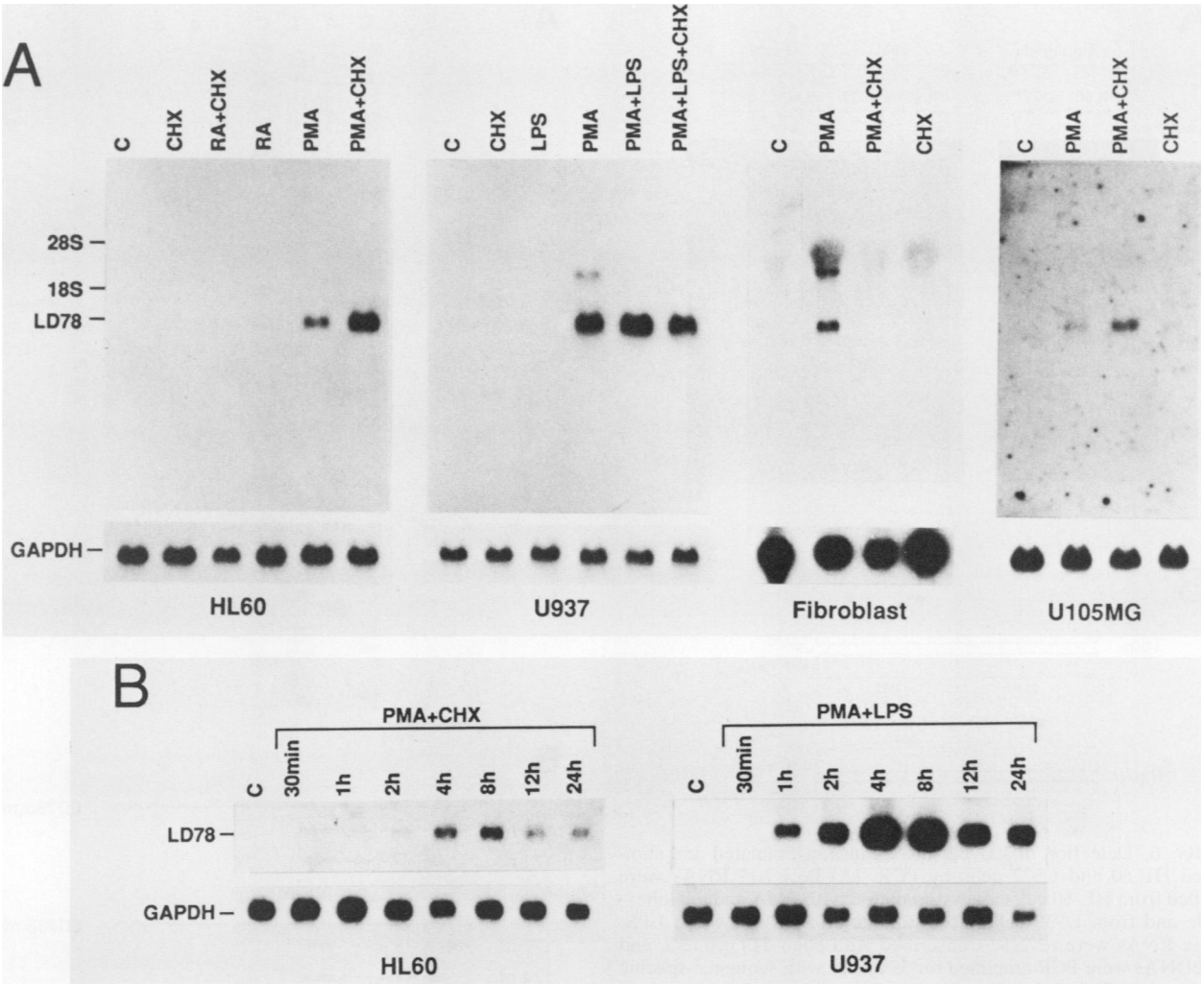

FIG. 5. Regulation of LD78 mRNA expression in different cell types. Total cellular RNAs (10 μg each) were fractionated on a 1% formaldehyde-agarose gel, transferred onto nitrocellulose filters, and hybridized with the LD78 cDNA probe. (A) RNAs were prepared from the human myeloid cell lines HL-60 and U937, primary culture of human fibroblasts, and human glioma cell line U105MG. The cells were stimulated for 5 h with cycloheximide (CHX) or retinoic acid (RA). c, Nonstimulated samples. (B) Time course of LD78 mRNA induction. HL-60 cells were stimulated with PMA and cycloheximide (CHX) and U937 cells were stimulated with PMA and LPS for 30 min to 24 h as indicated. After autoradiography, the probes were removed and the filters were rehybridized with the GAPDH cDNA probe. To estimate the levels of LD78 mRNAs, intensities of each signal were normalized from the signals of the GAPDH probe. The positions of 28S and 18S rRNAs are indicated.

were observed (Fig. 7A). We could not observe a typical half-nucleotide migration difference in this result, possibly because of the narrow spaces between the fragments of the M13 sequence ladder used as size markers and because of the different base compositions of the sequenced and elongated fragments. The elongated band corresponding to the 174-nucleotide fragment showed the strongest signal and may be a mixture of the LD78α major and the LD78β minor transcripts. On the basis of relative intensities of the bands corresponding to fragments of 171 and 177 nucleotides, which probably represent the LD78α minor and LD78β major transcripts, respectively, we assume that the level of the LD78β messages is lower than that of the LD78α messages. This result is consistent with the finding obtained by using the first method and again indicates that both the LD78β and LD78α genes are transcribed in stimulated U937 cells. The LD78α messages are, however, more abundant than the LD78β messages.

DISCUSSION

Characterization of human genes coding for LD78, a member of a new cytokine superfamily. We obtained evidence for at least three LD78-like genes in a human genome, the LD78α, LD78β, and LD78γ genes. We characterized two of them, the LD78α and LD78β genes. The former, present in the 4.2-kb *Eco*RI fragment, corresponded to the genomic DNA of the previously isolated LD78 cDNA (33); the latter, present in the 4.8-kb *Eco*RI fragment, showed 94% sequence homology with the LD78α gene. We also found that the LD78α gene exists as a single copy or a few copies per haploid genome, whereas the LD78β gene and the LD78γ gene present in the 6.5-kb *Eco*RI fragment vary in copy number among individuals (Fig. 1).

By mitogenic activation, Zipfel et al. (54) isolated and characterized a number of cDNA clones corresponding to the mRNAs inducible in human peripheral T cells. They

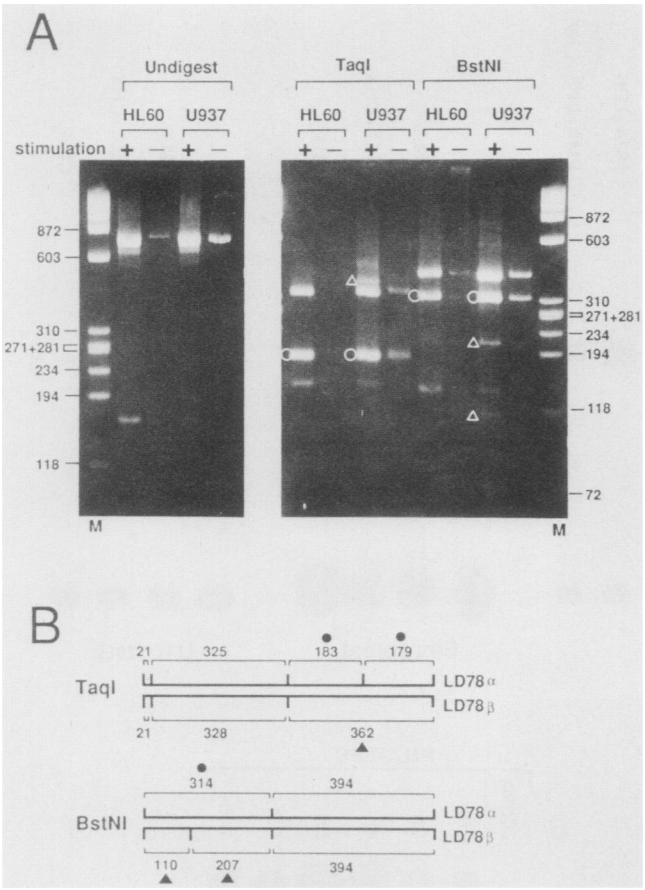

FIG. 6. Detection of LD78β mRNA in nonstimulated and stimulated HL-60 and U937 cells by PCR. (A) Poly(A)$^+$ RNAs were purified from HL-60 cells after stimulation with PMA and cycloheximide and from U937 cells after stimulation with PMA and LPS. These RNAs were reverse transcribed with oligo(dT) primers, and the cDNAs were PCR amplified for 35 cycles with sequence-specific primers (see Fig. 3 for locations of the primers). Products were digested with *Taq*I or *Bst*NI and fractionated on a 4% NuSieve GTG agarose gel. Fragmetns specific for LD78α and LD78β cDNAs are indicated by circles and triangles, respectively. Sizes (in base pairs) of a *Hae*III digest of φX174 replicative-form DNA (lanes M; Bethesda Research Laboratories) are indicated. (B) Predicted restriction fragments of amplified LD78α and LD78β cDNAs. Lines indicate the amplified cDNAs. The predicted lengths of the restriction fragments are shown above or below the lines. Because of its minute size, the 21-bp *Taq*I fragment is not visible on the gel.

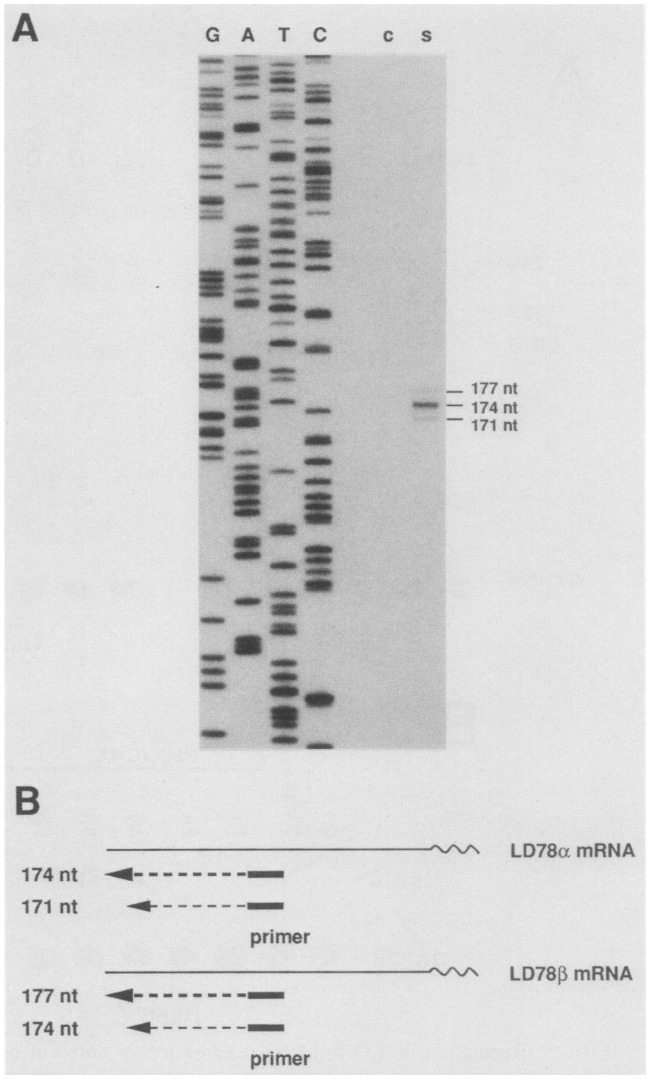

FIG. 7. Detection of LD78β mRNA in stimulated U937 cells by primer extension analysis. (A) A 5′-labeled synthetic oligonucleotide complementary to nucleotides 157 to 159 and 848 to 862 (according to the LD78α gene sequence; Fig. 3A) was hybridized to 3 μg of poly(A)$^+$ RNA from U937 cells stimulated with PMA and LPS for 5 h. Elongation and electrophoresis were carried out as described in the legend to Fig. 4. To determine the sizes of the reverse transcripts, sequencing reaction products of M13 DNA were coelectrophoresed. (B) Experimental strategy. Predicted reverse transcripts and their lengths are shown. Thick and thin dashed lines indicate the major and minor reverse transcripts, respectively. nt, Nucleotides.

found that one of their clones, pAT464, is identical with LD78 cDNA (53) and performed Southern blot analysis of the *Eco*RI-digested human placental DNA, using pAT464 cDNA as a probe. They observed 4.2- and 4.8-kb *Eco*RI fragments as well as two additional small fragments; one of them is approximately 2.3 kb long and the other is much smaller (53). Their observation suggests that in some individuals there is at least one LD78-like gene other than the three described here or that there are several restriction fragment length polymorphisms around some of the LD78β or LD78γ genes. We analyzed 14 genomic DNAs by Southern blot analysis; 12 of them were prepared from peripheral blood, and 2 were prepared from cultured human cell lines. We detected no such small fragments in any these genomic DNAs (Fig. 1). From murine T cells, Kwon and Weissman (20) isolated a cDNA clone and named it L2G25B. It proved to be identical with the cDNA encoding MIP-1α (10). They performed Southern blot analysis of the *Eco*RI-digested genomic DNAs extracted from C57BL/6 and BALB/c mice, using L2G25B cDNA as a probe, and noted the presence of a single restriction fragment of approximately 15 kb in length. They concluded that in the mouse genome there is only one L2G25B gene, a counterpart of the human LD78α gene. In contrast to our results shown in Fig. 1, we reported that the 4.8-kb *Eco*RI fragment is the single band detectable in the Southern blot analysis of an *Eco*RI-digested human placental DNA, using the LD78 cDNA as a probe (33). At present, we think that this result is probably due to a genomic DNA containing multiple copies of the LD78β gene

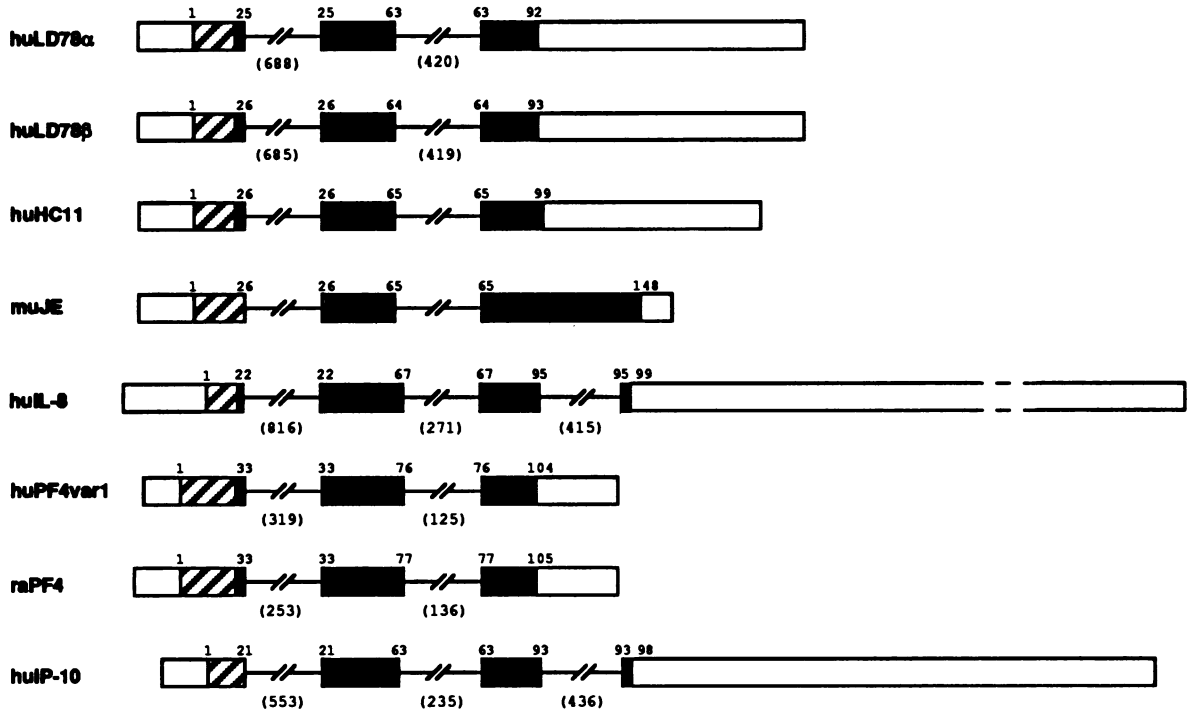

FIG. 8. Genomic structures of LD78α (huLD78α) and LD78β (huLD78β) genes and those of the several members of the new cytokine superfamily. Hatched and filled boxes indicate the leader sequence and mature protein-coding regions, respectively, with the number of amino acid residues. Open boxes denote the 5′ and 3′ noncoding regions. Introns and their lengths (in nucleotides) are shown by lines. Structures are derived from sequence data for human gamma interferon-inducible gene HC11 (huHC11) (6), murine platelet-derived growth factor-inducible gene *JE* (muJE) (18, 36), human IL-8 (huIL-8) (28), human platelet factor 4 variant 1 (huPF4var1) (15); rat platelet factor 4 (raPF4) (13), and human γ-interferon-inducible protein IP-10 (huIP-10) (22).

and a single copy of the LD78α gene rather than that the human placental DNA contains no LD78α gene but one copy of the LD78β gene.

Multiple copies of the LD78β gene may have been generated by DNA duplication and subsequent unequal crossing-over events. Similar events have been proposed to explain the variation in copy numbers of the visual pigment genes observed among normal individuals. The human red and green visual pigment genes have 98% sequence identity and are located on chromosome X in a head-to-tail tandem array. Variation in gene number has been observed only in the green pigment gene, and the arrangement of these pigment genes (i.e,. the red pigment gene is located at the 5′ edge of the array) explains the lack of copy number variation in this gene (30, 31, 46). Experiments to determine the arrangement of LD78 genes on human chromosomes are in progress in our laboratory.

Genomic organizations of several other members of the new cytokine superfamily have recently been determined (6, 13, 15, 18, 22, 28, 36), and their structures are schematically summarized in Fig. 8. These genes are organized from three exons and two introns except for the human IL-8 and IP-10 genes, both of which are composed of four exons and three introns. The first and second intron positions are also conserved among the members, and their first introns separate the leader peptides from the mature proteins. These similarities suggest that genes of the superfamily members were generated by duplication events from a common ancestral gene.

Putative regulatory sequences of the LD78 genes. In the 5′-flanking regions of the LD78α and LD78β genes, we found several potential regulatory sequences. The first was a decanucleotide sequence similar to a lymphokine consensus sequence, GAGPuTTCCAPy, and was identified at nucleotide position −60 of the LD78α and LD78β genes (Fig. 9A). This sequence has been identified in several lymphokine-cytokine genes, such as human and mouse granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor, and IL-3 genes (5, 32, 40, 45) (Fig. 9A). The sequence present in the mouse GM-CSF gene was seen to mediate an inducible response to the *tax trans*-activator encoded by the human T-cell leukemia virus type I (HTLV-I) but not to mediate a similar response to PMA (26). The second putative regulatory sequence, TGTG-GTCACCA, was located at nucleotide position −137 of the LD78α gene and at position −139 of the LD78β gene (Fig. 9B). Similar sequences have been detected in the promoter regions of several other cytokine genes, such as those encoding human and mouse GM-CSF and human γ-interferon (32) (Fig. 9B). A similar sequence was also present in the promoter region of the mouse *JE* gene, a member of the new cytokine superfamily of genes (36).

The third sequence, a $CC(A/T)_6GG$ element, was located far upstream from the first and second sequences at position −669 of the LD78α gene and at position −985 of the LD78β gene. This element has been implicated in tissue specific transcription of the actin genes (41) and in the serum response of the c-*fos* proto-oncogene (43, 44). Cross et al. (9) reported that a similar sequence located immediately downstream of the *trans*-acting factor NF-κB-binding site in the

A

huLD78α	CAGATTCCAT	-60
huLD78β	CAGATTCCAT	-60
huGM-CSF	GAGATTCCAC	-100
muGM-CSF	GAGATTCCAC	-107
huG-CSF	GAGATTCCAC	-189
muG-CSF	GAGATTCCCC	-192
huIL-3	GAGGTTCCAT	-126
huIL-3	GAGATCCCAC	-333
muIL-3	GAGGTTCCAT	-114
muIL-3	GAGATTCCAC	-295
consensus	GAGRTTCCAY	

B

huLD78α	TGTGGTCACCA	-137
huLD78β	TGTGGTCACCA	-139
huGM-CSF	TGTGGTCACCA	-57
muGM-CSF	TGTGGTCACCA	-64
huγ-IFN	ATCTGTCACCA	-93
muJE	TGTGGTCACAG	-286
consensus	TGTGGTCACCA	

FIG. 9. Putative regulatory sequences present in the 5'-flanking regions of the LD78α and LD78β genes and other cytokine genes. (A) Decanucleotide lymphokine consensus sequence; (B) conserved cytokine sequence. Nucleotide sequences are taken from the following references: human (hu-) and murine (mu-) GM-CSF (25); human granulocyte colony-stimulating factor (G-CSF) (29); murine G-CSF (45); human (IL-3) (52); murine IL-3 (27); human γ-interferon (γ-IFN) (14); murine *JE* (36). The position of the 5' end of the sequence with respect to the transcription initiation site or the 5' end of the cDNA is indicated at right of each sequence. R, Purine; Y, pyrimidine.

promoter region of the human IL-2 receptor α gene activates a heterologous promoter in PMA-stimulated Jurkat cells and in HTLV-I-infected MT-2 cells. They suggested that this sequence acts as a T-cell-specific positive regulatory element. In relation to this observation, Zipfel et al. (53) and our group (51) have found a high level of constitutive expression of the LD78 mRNAs in HTLV-I-infected T cells.

The fourth sequence was an *Alu* element of short interspersed highly repetitive sequences, inserted at position −299 from the putative transcription initiation site of the LD78β gene (Fig. 3A). This *Alu* sequence was 86% homologous to the *Alu* consensus sequence (Fig. 3B) and belongs to the d branch of the *Alu*-S subfamily (17). A similar sequence was not present in the corresponding region of the LD78α gene. All of the LD78β genes must be associated with the *Alu* element, since we detected no 4.5-kb *Eco*RI fragment expected for the LD78β gene without the *Alu* element, using Southern blot analysis (Fig. 1).

A negative regulatory element was identified within a member of the African green monkey *Alu* family (37). This 38-bp-long element was shown to reduce, but not to eliminate, expression of the simian virus 40 early and human β-globin promoters in a manner independent of orientation and position and hence was termed a reducer. Since the level of LD78β mRNA was lower than that of LD78β mRNA in PMA-stimulated U937 cells (Fig. 6 and 7), we speculate that the reducerlike element in the inserted *Alu* element is functional. Even if this *Alu* element lacks the reducer activity, it separates one of the putative regulatory sequences from the other two sequences and thus may disturb cooperative action of the transcription factors. All of these putative regulatory sequences except for the *Alu* element are conserved in LD78α and LD78β genes; hence, the expression of these two genes is probably regulated in the same manner.

Induction of LD78 gene expression. We found that LD78 gene expression can be induced in various cell types by stimulation with PMA. However, Zipfel et al. (53) observed that PMA stimulation alone does not induce the LD78 (AT464) mRNA in peripheral T cells and in the human T-cell line Jurkat and that simultaneous stimulation with PMA and phytohegglutinin is required for optimal LD78 (AT464) mRNA expression in those cells. Furthermore, the addition of cycloheximide during this stimulation led to a marked inhibition of LD78 mRNA expression in the primary culture of fibroblasts (Fig. 5A) and in Jurkat cells (53). Conversely, the addition of cycloheximide significantly increased the levels of LD78 mRNAs in HL-60 and U105MG cells, and in U937 cells it did not inhibit the response to PMA (Fig. 5A). These results suggest that LD78 mRNA expression is regulated differently in different cell types and that de novo protein synthesis is required for the induction of LD78 gene expression in fibroblasts and Jurkat cells. However, in myeloid HL-60 and U937 cells and glioma U105MG cells, the regulatory factors required for LD78 mRNA expression are present before stimulation, and a labile repressor may be involved in regulatory expression of the LD78 genes. Further experiments are under way to determine the regulatory mechanisms involved in LD78 gene expression.

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