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Characterization of Cytokine LD78 Gene Promoters: Positive and Negative Transcriptional Factors Bind to a Negative Regulatory Element Common to LD78, Interleukin-3, and Granulocyte-Macrophage Colony-Stimulating Factor Gene Promoters

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Cytokine LD78 is a human counterpart of the mouse macrophage inflammatory protein 1α/hematopoietic stem cell inhibitor. Promoters of the LD78α and LD78β genes showed similar inducible activities in two leukemic cell lines, K562 and Jurkat, but the induction mechanisms differed between the two cell lines. Further characterization of the LD78α promoter indicated that multiple positive and negative regulatory elements are present, some of which are differentially required for induction and repression of the promoter activity in different cells. One of the negative regulatory elements, ICK-1, functioned in both cell lines in the absence and presence of stimulation and was shown to be a recognition site for positive and negative transcriptional factors. This ICK-1 element contained a direct repeat, and similar repeats were also found in the negative regulatory elements of hematopoietic growth factor interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) gene promoters. Nuclear extracts from K562 and Jurkat cells formed several protein-DNA complexes with the LD78α ICK-1 element, one of which was also observed with the IL-3 and GM-CSF ICK-1 elements. Results from in vivo and in vitro analyses suggested that the protein forming this complex functions as a negative factor. The binding affinity of this protein, ICK-1A, to the LD78α ICK-1 element was low and was significantly affected by the incubation temperature and the salt concentration in the binding buffer. ICK-1B, another protein bound specifically by the LD78α ICK-1 element, was shown to be a positive factor important for induction of the promoter. These results suggested that ICK-1A plays an important role in balanced expression of LD78, IL-3, and GM-CSF during hematopoietic cell growth and differentiation.

Human LD78 (48), also known as AT464 (69) or G0S19S (2), is a member of a large superfamily of small secreted cytokines involved in cell growth and inflammation (18, 49, 54). The mouse counterpart of LD78 is macrophage inflammatory protein 1α (MIP-1α) (8), also known as hematopoietic stem cell inhibitor (19) or L2G25B (30). LD78 shows 74% amino acid sequence identity with MIP-1α. By using purified recombinant proteins, both LD78 and MIP-1α have been shown to suppress proliferation of hematopoietic stem and immature progenitor cells in vitro (3, 5, 19, 58) and in vivo (11, 32). MIP-1α has also been shown to enhance proliferation of more differentiated progenitor cells in vitro (5). In addition, LD78 has been found to stimulate osteoclast differentiation in a rat bone marrow culture system (29). As well as modulating proliferation and differentiation of hematopoietic cells, MIP-1α affects macrophage function in an autocrine manner (12), and LD78 is chemotactic for T lymphocytes (63).

Three distinct LD78 genes, clustered on chromosome 17 (21, 23), have been identified (2, 23, 43). The LD78α and LD78β genes both consist of three exons and share 94% sequence similarity. The 5′-flanking regions are also highly homologous over 2 kb. One characteristic structural difference is the insertion of a repetitive *Alu* sequence in the 5′-flanking region of the β gene at position −294 (relative to the transcription initiation site) (2, 43). The α gene exists as a single copy in each individual, but the number of copies of the β gene varies (21). Previously isolated LD78 cDNA is the cDNA copy of the LD78α gene transcript (2, 48, 69), and the β gene has been demonstrated to be active by the reverse transcriptase-polymerase chain reaction method (43) and by the isolation of LD78β cDNA (23). The proteins encoded by the two genes differ by three amino acid residues in mature secreted forms. No differences between the biological activities of recombinantly produced α and β proteins have been observed so far (29, 63). The third gene, LD78γ, is a pseudogene lacking most of the first intron and its upstream region. This pseudogene is found only in some individuals and, like the β gene, is variably amplified on the chromosome (21). In contrast to multiple LD78 genes in the human, there is only one MIP-1α gene in the mouse genome (30, 65).

A number of different cell types can be induced to express LD78 or MIP-1α mRNA upon stimulation (6, 30, 36, 43, 55, 69). However, the mechanism of induction of LD78 gene expression depends on the cell type (43, 43, 69). In monocytic cells and fibroblasts, LD78 mRNA, which is probably a mixture of LD78α and -β mRNAs, is induced by treatment of phorbol 12-myristate 13-acetate (PMA), while in peripheral blood T cells and T-cell leukemic Jurkat cells, phytohemag-

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glutinin (PHA) but not PMA induces LD78 mRNA. In fibroblasts and T cells, de novo protein synthesis is required for induction. In other cell types, transcriptional factors required for LD78 mRNA expression are present prior to stimulation. LD78 mRNA appears about 1 to 2 h after stimulation, reaches maximal levels 4 to 8 h after stimulation, and then gradually decreases (39, 43, 68, 69). The 3′ untranslated regions of the LD78α and -β mRNAs contain AU-rich sequences, which have been implicated in controlling the stability of cytokine mRNAs (7, 57) and therefore may partly involve postinduction repression of LD78 mRNA transcription. In some freshly isolated hematopoietic tumor cells and some cell lines, in contrast, LD78 mRNA is constitutively expressed (26, 68).

To elucidate the different induction mechanisms of LD78 gene expression in different cell types, we have characterized LD78α and -β gene promoters in two leukemic cell lines, K562 and Jurkat. In the course of these analyses, we identified a negative regulatory element that is active irrespective of stimulation. The element was found to be a target site for positive and negative transcriptional factors having different binding affinities.

MATERIALS AND METHODS

Construction of plasmids. For construction of 5′-deleted mutants of the LD78α promoter, a plasmid containing the *Bam*HI-*Xho*I fragment (from −1.2 kb to +54 bases, relative to the transcription initiation site) of the LD78α promoter region derived from the Lm LD-3 clone (43) was linearized at the *Bam*HI site. The DNA was then partially digested with exonuclease BAL 31 (Takara Shuzo, Kyoto, Japan) for various lengths of time, treated with DNA polymerase I large fragment (Takara) to form blunt ends, and digested with *Xho*I. The resulting fragments were fractionated on a 1% low-melting-point agarose gel, excised from the gel, and ligated into the *Sma*I-*Xho*I sites of pSP72 (Promega Biotec, Madison, Wis.). DNAs were transfected into *Escherichia coli* SURE (Stratagene, La Jolla, Calif.). After determination of the 5′-deleted ends by dideoxynucleotide sequence analysis (53), the fragments were excised with *Bgl*II and *Xho*I. The deleted fragments and the original *Bam*HI-*Xho*I fragment were inserted into the *Bgl*II-*Xho*I sites, located upstream of the bacterial chloramphenicol acetyltransferase (CAT) gene, of pBLCAT3 (33). The *Bam*HI-*Xho*I fragment of the LD78β promoter in the Lm LD-1 clone (43) and the *Hin*dIII (the site in the vector)-*Xho*I fragment of the LD78α gene in the cosLD78-7 clone (21) were also inserted into the *Bgl*II-*Xho*I and *Hin*dIII-*Xho*I sites, respectively, of pBLCAT3. A repetitive *Alu* sequence deletion mutant of the LD78β gene promoter (LD78β:Δ*Alu*) has a deletion between the *Eco*T22I (−633) and *Bst*XI (−351) sites.

For construction of 3′-deletion mutants, one of the 5′-deletion mutants in pSP72, containing a region from −408 to the *Xho*I site, was linearized at the *Xho*I site, variously deleted as described above, and digested with *Kpn*I. The resulting fragments were reinserted into the *Kpn*I-*Eco*RV sites of pSP72. After sequencing, the fragments were excised with *Hin*dIII and *Bgl*II digestion and cloned into the *Hin*dIII-*Bam*HI sites, located upstream of the thymidine kinase (*tk*) promoter-CAT gene, of pBLCAT5 (51).

Single-base-pair mutations were introduced into the LD78α promoter by site-directed mutagenesis (28), using the Mutan-G system (Takara). The *Kpn*I-*Eco*RI fragment of one of the 5′-deletion mutants in pBLCAT3, containing the LD78α promoter region from −194 to +54 and a portion of the CAT gene, was cloned into M13tv18, and mutagenesis was carried out according to the protocol provided by the supplier. The synthetic oligonucleotides used were 24 nucleotides long, a single-point mutation being incorporated in the middle position. The mutations were verified by sequence analysis, and the *Bam*HI-*Xho*I fragments were excised from the double-stranded M13 DNAs and reinserted into the *Bam*HI-*Xho*I sites of pBLCAT3.

Tetramers of ICK-1 elements were constructed essentially as described previously (15). Complementary synthetic oligonucleotides (see Fig. 5) were phosphorylated with T4 polynucleotide kinase, annealed, and ligated into tandem repeats by virtue of the presence at each end of an asymmetric *Ava*I restriction site. The ligation products were cloned into the asymmetric *Ava*I site in vector pGEM-AvaI. pGEM-AvaI is a derivative of pGEM-1 (Promega) in which the synthetic polylinker *Hin*dIII-*Xba*I-*Ava*I-*Sal*I-*Bam*HI has been inserted into the *Hin*dIII-*Bam*HI sites. The clones containing four tandem repeats of elements were obtained by minipreparations, and the sequence of the insert was verified by sequence analysis. The inserts were excised with *Hin*dIII and *Bam*HI and cloned into the *Hin*dIII-*Bam*HI sites of pBLCAT5 or pBL2SVE (50).

Cell culture. The human erythroleukemic cell line K562 and the T-cell leukemic cell line Jurkat were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and W. C. Greene, Duke University (Durham, N.C.), respectively, and grown in RPMI 1640 medium (GIBCO Laboratories, Grand Island, 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 at 37°C in humidified air with 5% CO_2.

DNA transfection. Transient transfections were carried out by the DEAE-dextran method (34). Approximately 2×10^7 cells were washed twice with serum-free RPMI 1640 medium, suspended in 1 ml of medium containing 0.1 M Tris-HCl (pH 7.4), 500 μg of DEAE-dextran (Pharmacia LKB Biotechnology, Uppsala, Sweden), and 16 μg of plasmid DNA (purified by two CsCl-ethidium bromide density gradient centrifugations), and incubated at 37°C for 30 min. In some transfections (Fig. 4 and 11B), 2 μg of pRSV/L (9), a plasmid containing the firefly luciferase gene linked to the Rous sarcoma virus (RSV) long terminal repeat, was cotransfected as an internal control for transfection efficiency. After incubation, the cells were washed twice with serum-free medium, resuspended in the same medium containing 10% serum, and incubated at 37°C for 15 h. One half of the cell suspension was then treated with one or more of the following agents: PMA (Sigma Chemical Co., St. Louis, Mo.), at a final concentration of 50 ng/ml; PHA (Wellcome Diagnostics, Dartford, England), 1 μg/ml; and recombinant human tumor necrosis factor alpha (TNF-α; Genzyme Corp., Cambridge, Mass.), 2,000 U/ml. The other half was used as an unstimulated control. Cells were incubated for another 10 h. Transfections were repeated three times with two different plasmid DNA preparations.

CAT and luciferase assays. The CAT assay was performed as described previously (17). Following transfection, the cells were harvested, rinsed with phosphate-buffered saline, resuspended in 100 μl of 0.1 M Tris-HCl (pH 7.8), and lysed by sonication. After centrifugation, the supernatant was collected, and the protein concentration was determined by the Bradford assay (4). For one assay, 150 μg of extract was used with 0.25 mCi (1 Ci = 37 GBq) of [^{14}C]chloramphenicol (50 mCi/mmol; Dupont, NEN Research Products, Boston, Mass.) in 75 μl of 0.25 M Tris-HCl (pH 7.8)–0.5 mM acetyl

coenzyme A (Pharmacia). After incubation at 37°C for 2 h, chloramphenicol and its derivatives were extracted with ethyl acetate. The ethyl acetate phase was dried under vacuum, and the residue was resolved in 20 μl of ethyl acetate. The acetylated forms of chloramphenicol were separated by chromatography on silica gel thin-layer plates (Merck, Darmstadt, Germany), which were developed in 95% chloroform–5% methanol. The plates were autoradiographed or scanned with a Bio-Image Analyzer (Fuji Film, Kyoto, Japan). CAT activities were normalized with regard to the molar content of the transfected plasmid.

The luciferase assay was carried out by using a PicaGene assay kit (Toyo Ink., Tokyo, Japan). For one assay, 100 μg of extract was mixed with 100 μl of reaction buffer supplied in the kit, and the chemiluminescence was counted by a scintillation counter for 1 min.

Preparation of nuclear extracts. Crude nuclear extracts were prepared by the procedure of Dignam et al. (10). The extracts were dialyzed against buffer containing 20 mM Tris-HCl (pH 7.9), 20% (vol/vol) glycerol, 50 mM KCl, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol at 4°C for 4 h, frozen as aliquots in liquid nitrogen, and stored at −80°C.

DNase I footprinting analysis. DNase I footprinting analysis was carried out essentially as described previously (16, 25). Upper and lower strands of the LD78α promoter region were labeled by digesting one of the 5′-deletion mutants in pBLCAT3, plasmid −194, with *Bam*HI and *Xho*I, respectively, followed by phosphorylation with [γ-^{32}P]ATP and polynucleotide kinase. After recleavage with *Xho*I and *Bam*HI, respectively, the fragments containing singly end-labeled promoter region were purified by electrophoresis. Binding reactions were carried out at 0°C for 30 min in a final volume of 40 μl containing 20 mM *N*-2-hydroxyethylpiperadine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 30 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 0.2 μg of poly(dI-dC), 25 μg of nuclear extract, and 10^5 cpm of end-labeled DNA fragment (equivalent to about 5 ng). Before addition of the labeled fragment, the mixtures were kept at 0°C for 10 min. Following the binding reaction, 4 μl of freshly diluted precalibrated DNase I (Worthington Biochemical Corp., Freehold, N.J.) solution in 66 mM $CaCl_2$ was added, and digestion was carried out at 30°C for 60 s. The reactions were terminated by adding 100 μl of stop solution containing 1% sodium laurylsulfate, 20 mM EDTA, 200 mM NaCl, and 100 μg of tRNA per ml, extracted with phenol and chloroform, and precipitated with ethanol. The samples and the products of G+A sequencing reaction (38) prepared by using the same labeled fragments were coelectrophoresed on 6% sequencing gels.

Gel retardation assay. The gel retardation assay was performed essentially as described previously (14, 60, 61). Annealed oligonucleotides used as probes were the same as one copy of the ICK-1 tetramers except for the LD78α ICK-1 wild-type probe, which does not contain four-base overhangs at each end. The oligonucleotides were labeled at one end, electrophoresed on 10% polyacrylamide gels, and purified by electrophoresis. Unless otherwise stated, the binding reactions were carried out in a 20-μl reaction mixture containing 20 mM HEPES-KOH (pH 7.9), 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10% (vol/vol) glycerol, 5,000 cpm (equivalent to about 0.2 ng) of various probes, 0.2 μg of poly(dI-dC) (Pharmacia), and 5 μg of nuclear extract at 0 or 30°C for 30 min. In some experiments, 0.4 μg of poly(dA-dT) (Pharmacia) was added. Before addition of the probe, the mixture was kept at 0°C for 5 min. In competition experiments, cold annealed oligonucleotides were added with the nonspecific competitor poly(dI-dC). After incubation, samples were fractionated on 6% polyacrylamide gels (acrylamide/bisacrylamide, 40:1) containing 12 mM Tris acetate (pH 7.5) and 1 mM EDTA at 4°C with buffer circulation.

Methylation interference assay. Methylation interference analysis was carried out as described previously (67). Annealed oligonucleotides labeled at one end were methylated with dimethyl sulfate (38). The preparative binding reaction was performed as described above with approximately 5×10^5 cpm of methylated DNA and 200 μg of nuclear extracts. The complexed and free probes were separated on 6% acrylamide gels, electroeluted, and cleaved with piperidine. To clearly separate LD78α ICK-1 complexes B and C, about one-fourth of the gel from the bottom end was cut out for eluting free probes when the dye had nearly reached bottom of the gel, and electrophoresis was continued for another 30 min. The samples and the sequencing reaction products (G and G+A) of the original oligonucleotides were coelectrophoresed on 20% sequencing gels.

RESULTS

Promoter activities of LD78α and -β genes in K562 and Jurkat cells. Fragments of the 5′-flanking regions of the LD78α and -β genes, −1.4 kb to +54 bases (α gene) and −1.7 to +54 bases (β gene), were placed upstream of the promoterless CAT gene in the expression vector pBLCAT3. These plasmids were transiently transfected into the erythroleukemic cell line K562 and the T-cell leukemic cell line Jurkat. K562 cells differentiate into cells with megakaryocytic characteristics in response to PMA stimulation (62).

After transfection, cells were left unstimulated or stimulated with one or more of the agents PMA, TNF-α, and PHA. Promoter activities were determined by the CAT assay, using crude cell extracts. An autoradiogram of the CAT assay of one representative experiment is shown in Fig. 1. In K562 cells, both α and β promoters were activated to approximately equal extents by PMA treatment and enhanced about threefold by addition of TNF-α, although TNF-α alone did not activate the promoters. In Jurkat cells, simultaneous addition of PHA and PMA stimulated both promoter activities about twofold compared with PHA treatment alone, but PMA treatment alone did not activate the promoters. In the absence of stimulation, both promoters were inactive in both cell types.

The β-promoter region contains, at −294, a repetitive *Alu* sequence about 300 bp long in an inverse orientation relative to transcription of the β gene (43). This sequence is not found in the corresponding region of the α gene. The *Alu* sequence has been reported to function as a negative regulatory element in transcription (52, 64). We therefore deleted most of the *Alu* sequence in the β-gene promoter–CAT construct. The *Alu* deletion mutant, LD78β:Δ*Alu*, has approximately the same promoter activity as does the wild-type plasmid when stimulated with PMA (K562) or with PHA-PMA (Jurkat). The mutant still contains 36 bp of the *Alu* sequence, a portion proximal to the transcription start site, but it does not contain the transcriptional silencer element in the *Alu* sequence (64). This finding suggested that the *Alu* sequence in the β promoter has no negative regulatory activity on induction of β-gene expression.

These results indicate that the transcriptional regulatory mechanisms of the α and β genes differ in K562 and Jurkat cells but that the two promoters are similarly regulated.

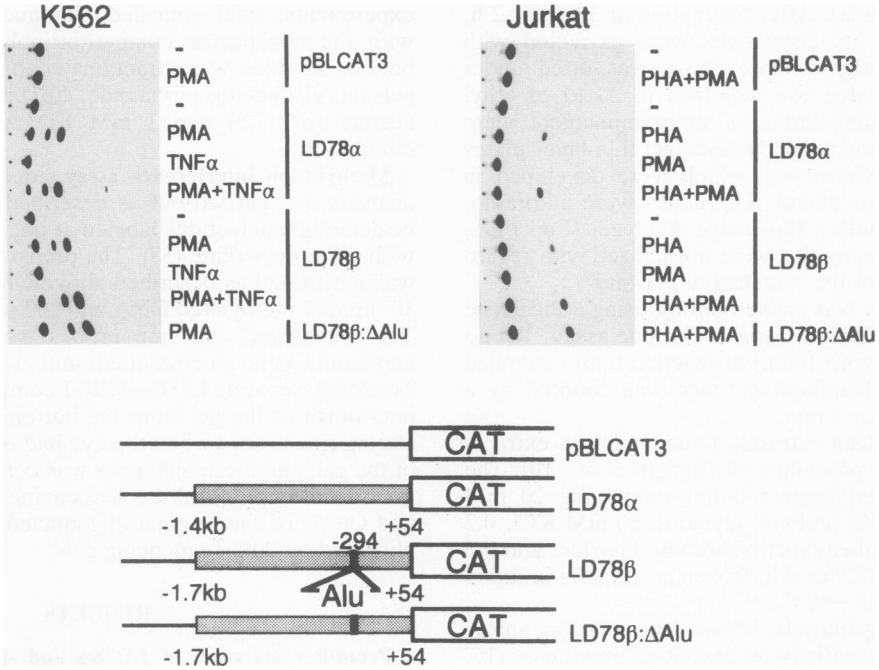

FIG. 1. CAT activity after transfection of K562 and Jurkat cells with LD78α and LD78β promoter-CAT constructs and induction with different stimuli. *Bam*HI-*Xho*I fragments of LD78α and -β genes, −1.4 kb to +54 bases (α gene) and −1.7 kb to +54 bases (β gene), were linked to the promoterless CAT reporter gene in the vector pBLCAT3. A deletion mutant of repetitive *Alu* sequence in the β-gene promoter, LD78β:Δ*Alu*, was also constructed. These constructs and the vector pBLCAT3 were transiently transfected into K562 and Jurkat cells by the DEAE-dextran method, and the cells were subsequently stimulated for 10 h with various agents as indicated. One representative CAT assay for each cell types is shown. At the bottom, constructions are schematically represented.

Positive and negative regulatory sequences which are differentially required for LD78α promoter activation and repression in K562 and Jurkat cells. To identify DNA sequences within the 5′-flanking region of LD78α gene that are required for its induction in K562 and Jurkat cells, sequential 5′-deletion mutants of upstream sequences were constructed. A plasmid containing a 5′-flanking region of 4.5 kb, derived from an LD78α cosmid clone (21), was also constructed and included in the series of mutants. These plasmids were transfected into K562 and Jurkat cells, and subsequently the cells were stimulated with PMA (K562) or with PHA-PMA (Jurkat).

The promoter activity of each plasmid is shown in Fig. 2A. Deletion of sequences up to −173 had no apparent effect in K562 cells, while in Jurkat cells, deletion from −327 to −194 resulted in a twofold increase in promoter activity. Removal of 24 additional nucleotides, from −173 to −150, had a detrimental effect in Jurkat cells. In contrast, the same deletion decreased the CAT activity from 115% to 42% in K562 cells, but deletion of an additional 15 or 16 nucleotides (plasmid −134 or −133) returned the level of promoter activity to that seen with plasmid −173. Plasmid −112 (K562) or −149 (Jurkat) and further deletions had almost no promoter activity. In the absence of stimulation, the promoter activities of all plasmids were 2% or lower, almost the same as that of the vector in unstimulated or stimulated cells (data not shown).

To further delineate the regulatory sequences in the LD78α promoter region, various 3′ deletions with fixed 5′ ends (at position −408) were placed in front of the *tk* promoter in pBLCAT5. The results (Fig. 2B) showed similar patterns of promoter activities in K562 and Jurkat cells. Upon stimulation, deletion from −47 to −60 decreased the promoter activity significantly. Plasmids −71 and −87 had virtually no activity. When the deletion was extended to −94, the activity increased to 17 and 37% in K562 and Jurkat cells, respectively, compared with the activity of plasmid −47. Removal of an additional 14 nucleotides again reduced the activity to less than 1% (K562) or 3% (Jurkat), the same level as that of the vector. Without stimulation, only plasmid −95 retained detectable activity, 4 and 10% in K562 and Jurkat cells, respectively.

These results suggest that positive regulatory sequences are present in the LD78α promoter region between −173 and −149, −133 and −112, −47 and −71, and −95 and −109. The results also suggest the existence of negative regulatory sequences. Negative regulatory sequences, located between −327 and −194 and between −149 and −134, functioned only in Jurkat and K562 cells, respectively. These sequences were active at least in stimulated cells, as no apparent increase in CAT activity was observed in unstimulated cells. In contrast, another negative regulatory sequence between −87 and −95 functioned in both cell types with or without stimulation.

DNase I footprinting analysis and potential regulatory elements. To correlate the functional analyses described above with binding sites for potential *trans*-acting factors affecting the LD78α promoter, we carried out DNase I footprinting. Crude nuclear extracts were prepared from K562 and Jurkat cells unstimulated or stimulated for 1 or 5 h. Both the upper and lower strands of the LD78α promoter were subjected to partial DNase I digestion in the absence or presence of nuclear extracts (Fig. 3A); the results are summarized in Fig. 3B, which also shows potential regulatory elements.

Several regions were protected from DNase I digestion in the presence of nuclear extracts. Footprint A corresponded

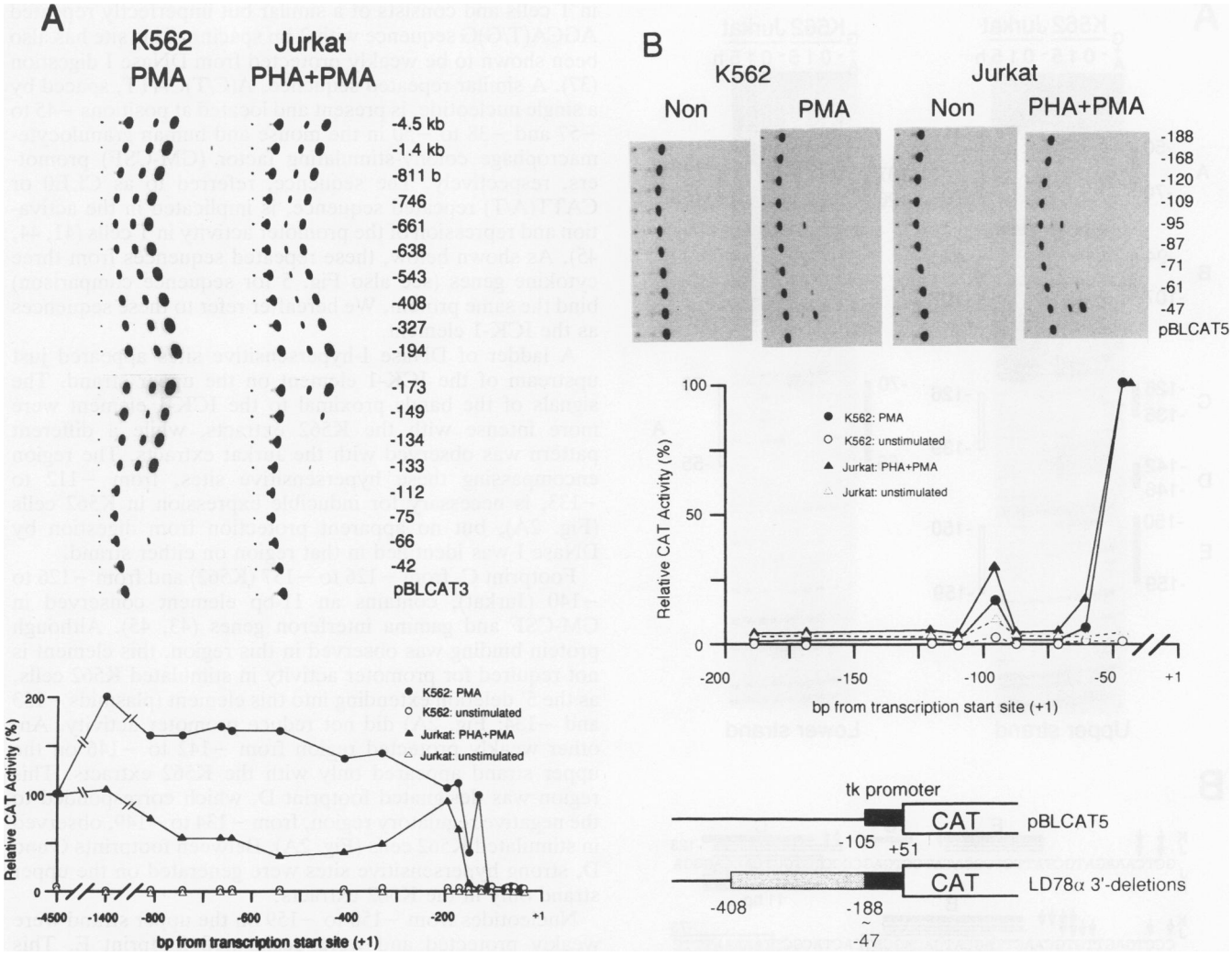

FIG. 2. Identification of positive and negative regulatory sequences in the LD78α promoter. Sequential 5′-promoter deletion (A) and 3′-promoter deletion (B) mutants were transfected into K562 and Jurkat cells as described for Fig. 1. After transfection, the cells were left unstimulated or stimulated with PMA (K562) or PHA-PMA (Jurkat). One representative CAT assay for each cell type is shown (for 5′-deletion mutants, only the result of transfection in the presence of stimulation is shown). Below the autoradiograms, the results are summarized graphically; the activity of the longest mutant in the corresponding cell type in the presence of stimulation is taken as 100%. Activities of the 5′-deletion mutants, plasmids −134 and −133, are the same and are indicated as a single circle or triangle. At the bottom of panel B, construction of 3′-deletion mutants is schematically represented. These mutants contain promoter fragments of various lengths linked to the *tk* promoter-CAT reporter gene in the vector pBLCAT5. 5′-deletion mutants were constructed as shown in Fig. 1.

to a region from −50 to −70 (K562) and a slightly larger region from −41 to −70 (Jurkat) and contains overlapping sequences similar to CK-1, also referred to as conserved lymphokine element 1 (CLE1), and interleukin-6 (IL-6) response elements, which are found in the promoter regions of several cytokines (13, 46, 56) and acute-phase protein genes (20, 24, 35, 66), respectively. Three nucleotides from the IL-6 response element, there is a purine-rich sequence similar to the binding site of proto-oncogene c-*ets* family proteins, which have been found associated with several cytokine genes (22, 27, 31). Adjacent to this element, there is an element similar to the P sequence which has been identified in the human IL-4 promoter and confers responsiveness of the gene to T-cell activation signals (1). However, the region encompassing the 5′ end of the purine-rich element and its upstream sequence of about 10 bp was not digested with DNase I; therefore, we could not determine whether a protein binds to the P-like element. Between this P-like element and purine-rich element, K562 extracts prepared from stimulated cells produced a DNase I-hypersensitive site on the lower strand which was much less prominent in Jurkat extracts. The promoter activities of the 3′-deletion plasmids −61 and −71 (Fig. 2B) indicated that some or all of these elements are required for inducible expression of the LD78α gene.

Footprint B, less pronounced and observed only on the upper strand, corresponded to nucleotides −94 to −107. The promoter activities of 3′-promoter deletion mutants, plasmids −95 and −109, suggested that this region contains positive and negative regulatory sequences functioning in both K562 and Jurkat cells (Fig. 2B). In this region, there is an element that shares significant sequence homology with the binding site of nuclear inhibitory protein (NIP), which has been identified in the human IL-3 promoter (37). This

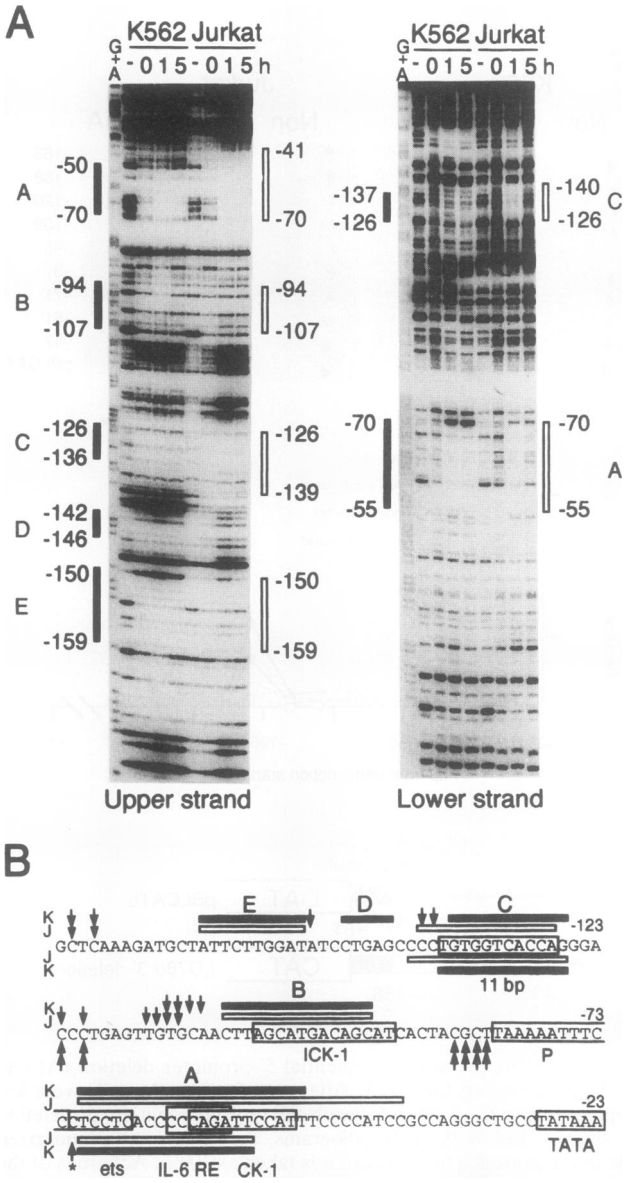

FIG. 3. DNase I footprinting over the LD78α promoter in K562 and Jurkat nuclear extracts. (A) DNase I footprinting analysis. Nuclear extracts were prepared from K562 and Jurkat cells unstimulated (0 h) or stimulated with PMA (K562) or PHA-PMA (Jurkat) for 1 or 5 h. Singly end-labeled upper or lower strands of LD78α promoter fragments were preincubated in the absence (−) or presence of extracts and digested with DNase I. A G+A sequence ladder of the same end-labeled fragment was electrophoresed in parallel. Closed (K562) and open (Jurkat) boxes indicate the footprints (A to E). The control lane without extract (−) in the middle of the left panel was less loaded than the other lanes (compare with the leftmost control lane). (B) Schematic representation of the results of DNase I footprinting. Only the upper-strand sequence is presented, along with potential regulatory elements. Footprints on the upper and lower strands are shown above and below the sequence, respectively. Sites rendered hypersensitive to DNase I digestion are indicated by arrows, and a stimulation-specific site is labeled with an asterisk. RE, response element.

element, located at positions −92 to −104, consists of a repeated AGCAT sequence with 3-bp spacing. The NIP-binding site of the IL-3 promoter, located at positions −249 to −262, has also been shown to be inhibitory for induction in T cells and consists of a similar but imperfectly repeated AGCA(T/G)G sequence with 2-bp spacing. This site has also been shown to be weakly protected from DNase I digestion (37). A similar repeated sequence, A(C/T)CATT, spaced by a single nucleotide, is present and located at positions −45 to −57 and −38 to −50 in the mouse and human granulocyte-macrophage colony-stimulating factor (GM-CSF) promoters, respectively. The sequence, referred to as CLE0 or CATT(A/T) repeated sequence, is implicated in the activation and repression of the promoter activity in T cells (41, 44, 45). As shown below, these repeated sequences from three cytokine genes (see also Fig. 5 for sequence comparison) bind the same protein. We hereafter refer to these sequences as the ICK-1 element.

A ladder of DNase I-hypersensitive sites appeared just upstream of the ICK-1 element on the upper strand. The signals of the bands proximal to the ICK-1 element were more intense with the K562 extracts, while a different pattern was observed with the Jurkat extracts. The region encompassing these hypersensitive sites, from −112 to −133, is necessary for inducible expression in K562 cells (Fig. 2A), but no apparent protection from digestion by DNase I was identified in that region on either strand.

Footprint C, from −126 to −137 (K562) and from −126 to −140 (Jurkat), contains an 11-bp element conserved in GM-CSF and gamma interferon genes (43, 45). Although protein binding was observed in this region, this element is not required for promoter activity in stimulated K562 cells, as the 5′ deletion extending into this element (plasmids −133 and −134; Fig. 2A) did not reduce promoter activity. Another weakly protected region from −142 to −146 on the upper strand appeared only with the K562 extracts. This region was designated footprint D, which corresponded to the negative regulatory region, from −134 to −149, observed in stimulated K562 cells (Fig. 2A). Between footprints C and D, strong hypersensitive sites were generated on the upper strand only in the K562 extracts.

Nucleotides from −150 to −159 on the upper strand were weakly protected and were designated footprint E. This region is required for induced expression of the LD78α gene in both K562 and Jurkat cells, but no conserved sequence among cytokine genes has been found.

The potential regulatory elements described above correlated well with the DNase I footprints. In addition, extracts prepared from unstimulated cells gave almost the same results as did extracts from stimulated cells, and no clear stimulation-dependent protein binding site was detected.

The ICK-1 negative regulatory element is the binding site for positive and negative transcriptional factors. We focused on the characterization of the inhibitory element, ICK-1, that functions in both K562 and Jurkat cells. Five single-base-pair mutations were introduced into this element (at positions −94, −96, −98, −100, and −102) by using the 5′-deletion mutant plasmid −194 (Fig. 4A). These mutants were constructed by site-directed mutagenesis, and their promoter activities were determined by the CAT assay (Fig. 4B and C).

In Jurkat cells, mutants M1 and M4 upregulated the level of induced expression, whereas mutant M5 reduced the level compared with the wild-type plasmid. Mutants M2 and M3 abolished or severely reduced the induction. These results suggest that the M1 and M4 mutations may have resulted in increasing binding of a positive factor to the element and that the other mutations may have inhibited this binding or increased the negative factor binding or both. The possibility that mutations M1 and M4 inhibited the binding of a negative

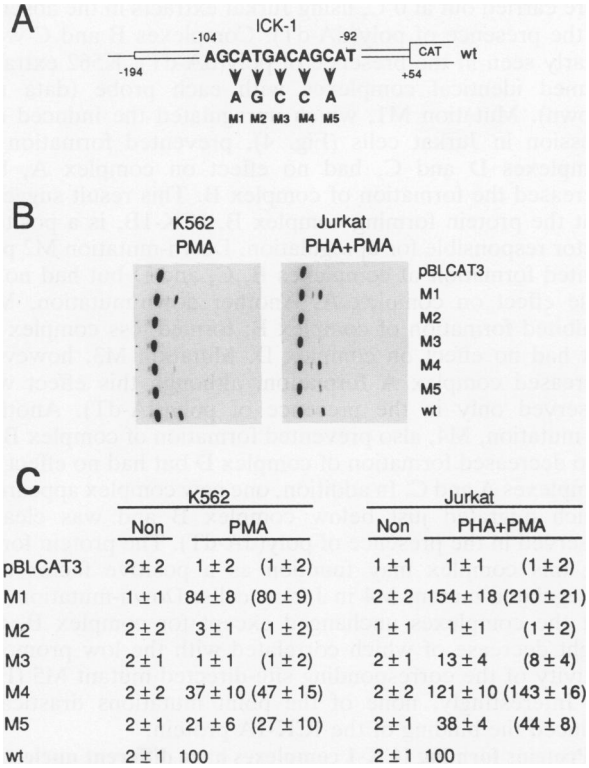

FIG. 4. Effects of single-base-pair mutations on LD78α promoter activity in K562 and Jurkat cells. (A) Locations of five point mutations in the LD78α ICK-1 element. These point mutations were introduced by site-directed mutagenesis, using a 5′-promoter mutant, plasmid −194 (Fig. 2A), as the template. wt, wild type. (B and C) Promoter activities of mutants M1 to M5. Transfection of K562 and Jurkat cells with mutant plasmids was performed as described for Fig. 1. The wild-type plasmid and the vector pBLCAT3 were also included in the transfection as controls. (B) Representative CAT assay; (C) Relative CAT activity of each mutant (mean of three experiments ± standard error), expressed as a percentage of the activity of the wild-type plasmid in the corresponding cell type in the presence of stimulation. Percentages shown in parentheses are those obtained from cotransfections with pRSV/L (9) and normalized against the level of luciferase expression.

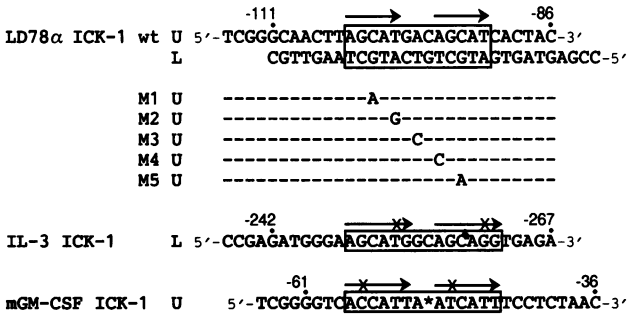

FIG. 5. Structures of LD78α, IL-3, and GM-CSF ICK-1 oligonucleotides. The nucleotide sequences of the double-stranded LD78α ICK-1 wild-type (wt) oligonucleotides are shown with the upper (U)-strand sequences of the single-base-pair mutants M1 to M5. Human IL-3 (lower [L] strand) and mouse GM-CSF (upper strand) ICK-1 oligonucleotides are also shown. Nucleotide sequences were taken from references 43 (LD78α), 59 (IL-3), and 40 (GM-CSF). The ICK-1 elements are boxed, and the direct repeats within the elements are indicated by arrows. The GM-CSF ICK-1 sequence is aligned with insertion of a gap (*) for maximum homology with the other two ICK-1 elements. The sequences shown are those of probes used in the gel retardation assay (Fig. 6, 8, and 10) and those that constitute the unit that was tandemly repeated four times (Fig. 11). In the gel retardation assay, blunt-ended oligonucleotides lacking overhangs of four nucleotides at both ends were used for the LD78α ICK-1 wild-type probe.

factor may be ruled out because in the absence of stimulation, these mutants had almost no activity, in contrast to one of the 3′-deletion mutants, plasmid −96, which slightly increased basal promoter activity (Fig. 2B). In addition, if a mutation inhibited binding of the negative factor but not the positive factor, the degree of upregulation would be increased more. These facts suggest that neither mutation severely affects the binding of the negative factor. A similar pattern of activities was observed in K562 cells, although the induced promoter activities of mutants M1 and M4 were less than that of the wild type.

In these CAT assays, the same amount of protein extract from each transfected cell sample was used, and the amount was adjusted to give a signal within the linear range. Furthermore, when an RSV-luciferase expression plasmid (9) was used as an internal control for transfection efficiency, approximately the same results were obtained (Fig. 4B). However, since RSV long terminal repeat is induced severalfold by PMA, normalization by luciferase activity was carried out only in the PMA stimulation experiment.

These results suggest that the ICK-1 negative regulatory element is recognized at least in Jurkat cells by positive and negative factors and that the positive factor(s) is essential for induction of the LD78α promoter. Therefore, the 3′-promoter deletion mutant, plasmid −109, which lacks the whole ICK-1 element, resulted in loss of promoter activity rather than upregulation, and plasmid −95, which is deleted of 3 bp of the 3′ end of the element, may have affected the binding of the negative factor but not the positive factor(s) (Fig. 2B).

The LD78α ICK-1 element binds several nuclear proteins in vitro, one of which also binds to the IL-3 and GM-CSF ICK-1 elements. To identify the positive and negative regulatory factors which bind to the LD78α ICK-1 element, oligonucleotides of the wild-type and mutant LD78α ICK-1 elements as well as those of the IL-3 and GM-CSF ICK-1 elements were synthesized. The sequences of the oligonucleotides are shown in Fig. 5. The 5′-end-labeled oligonucleotides used as probes were incubated with nuclear extracts, and the DNA-protein complexes formed were separated by electrophoresis on a 6% polyacrylamide gel. As for the wild-type LD78α ICK-1 probe, shorter oligonucleotides which lack four-nucleotide overhangs at each end were used, but the same binding complexes shown in Fig. 5 were observed with oligonucleotides with protruding ends (data not shown).

We first analyzed the binding reaction under different conditions, using nuclear extracts from stimulated Jurkat cells. Incubation of the labeled LD78α ICK-1 probe with extracts at 0°C for 30 min resulted in the formation of at least four complexes (Fig. 6A). The same binding pattern was observed with extracts from K562 cells (see Fig. 10; data not shown). Addition of poly(dA-dT) to the reaction mixture resulted in the disappearance of the slowest-migrating complex D. When the reaction was performed at 30°C for 30 min, complex A disappeared. The IL-3 and GM-CSF ICK-1 probes formed complexes with the same mobility as LD78α complex A. Their signals were more intense than that of LD78α complex A, 584% (IL-3 ICK-1) and 393% (GM-CSF ICK-1) compared with the LD78α complex A (100%) formed

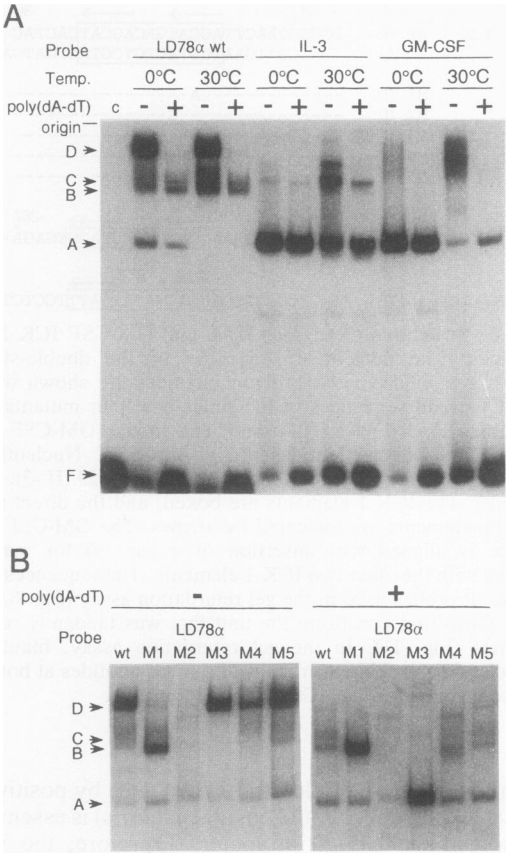

FIG. 6. Formation of DNA-protein complexes with use of LD78α, IL-3, and GM-CSF ICK-1 oligonucleotides. (A) Protein binding under different conditions. 5′-end-labeled ICK-1 probes (see Fig. 5) used in the gel retardation assay are indicated at the top. The binding reaction was carried out on ice (0°C) or at 30°C in the absence (−) or presence (+) of poly(dA-dT) with 5 μg of nuclear extracts prepared from Jurkat cells stimulated with PHA-PMA for 1 h. wt, wild type; c, LD78α ICK-1 probe without extract. Arrows at the right indicate the positions of ICK-1 complexes A to D and unbound probe (F). (B) Effects of single-base-pair mutations in the LD78α ICK-1 element on formation of complexes A to D. The binding reaction was performed at 0°C in the absence (−) or presence (+) of poly(dA-dT) with the same extracts as in panel A. The mutations which prevented complex formation are summarized in Fig. 7B.

in the absence of poly(dA-dT) at 0°C, but the intensities were reduced when the reactions were done at 30°C. The interaction of the protein with the ICK-1 elements was specific, as a complex with the same mobility was not observed with oligonucleotides of the granulocyte colony-stimulating factor CLE0 element, which is similar to the GM-CSF ICK-1 element but does not contain the repeated sequence (41) (data not shown). These results suggest that the repeated sequences bind the same protein forming ICK-1 complex A. Besides complex A, the IL-3 and GM-CSF ICK-1 elements formed complexes with high sensitivity to poly(dA-dT). One exception was one of the IL-3 complexes that migrated with LD78α complex C.

We then analyzed the effects of point mutations in the LD78α ICK-1 element on the formation of protein-DNA complexes (Fig. 6B). These mutations correspond to those of the site-directed mutants (Fig. 4). The binding reactions were carried out at 0°C, using Jurkat extracts in the absence or the presence of poly(dA-dT). Complexes B and C were clearly seen in the presence of poly(dA-dT). K562 extracts formed identical complexes with each probe (data not shown). Mutation M1, which upregulated the induced expression in Jurkat cells (Fig. 4), prevented formation of complexes D and C, had no effect on complex A, but increased the formation of complex B. This result suggests that the protein forming complex B, ICK-1B, is a positive factor responsible for upregulation. Down-mutation M2 prevented formation of complexes B, C, and D but had no or little effect on complex A. Another down-mutation, M3, inhibited formation of complex B, formed less complex C, but had no effect on complex D. Mutation M3, however, increased complex A formation, although this effect was observed only in the presence of poly(dA-dT). Another up-mutation, M4, also prevented formation of complex B; it also decreased formation of complex D but had no effect on complexes A and C. In addition, one new complex appeared, which migrated just below complex B and was clearly observed in the presence of poly(dA-dT). The protein forming this complex may function as a positive factor that upregulated mutant M4 in Jurkat cells. Down-mutation M5 left the complexes unchanged except for complex B, the slight decrease of which correlated with the low promoter activity of the corresponding site-directed mutant M5 (Fig. 4). Interestingly, none of the point mutations drastically reduced the binding of the ICK-1A protein.

Proteins forming ICK-1 complexes have different nucleotide requirements for binding. Gel retardation assays showed that each of the five point mutations affected the formation of complexes differently (Fig. 6B). We then studied further the nucleotide requirements of each protein for binding, using the methylation interference assay. Preparative binding reactions with methylated and singly end-labeled probes were carried out in the presence of poly(dA-dT) for analysis of the contact sites of the ICK-1A, -B, and -C proteins and, in the absence of poly(dA-dT), for that of the ICK-1D protein.

The interference patterns obtained with Jurkat extracts are shown in Fig. 7A. In the ladders of the lower strand, several fragments, especially the longer G-cleaved fragments, migrated as doublets, and some nonspecific bands of pyrimidine-cleaved fragments appeared. This result may be due to the secondary structure, and methylated A residues served to provide additional information for the contact sites. The patterns of the lower strand were those obtained with the longer oligonucleotides (Fig. 5), but the same unusual bands were observed with the shorter oligonucleotides used in the gel retardation assays (data not shown).

The contact region of the ICK-1A protein covered the entire ICK-1 element. Methylation of G or A nucleotides at positions −95, −99, and −103 on the upper strand and −94, −97, −100, and −102 on the lower strand significantly interfered with the binding of ICK-1A. Partial interference was also produced by methylation of nearby nucleotides at positions −91, −105, and −106 on the lower strand. These strong contact sites on the lower strand were identical with those obtained for NIP (ICK-1A), using the IL-3 ICK-1 sequence (37). Methylated nucleotides at positions −99 on the upper strand and at positions −97 and −100 on the lower strand strongly interfered with the binding of both ICK-1B and -C proteins, and those at positions −91 and −94 on the lower strand and at positions −95 and −103 on the upper strand partially interfered with the binding of both proteins and only the ICK-1B protein, respectively. All of these contact sites are also important for binding of the ICK-1A

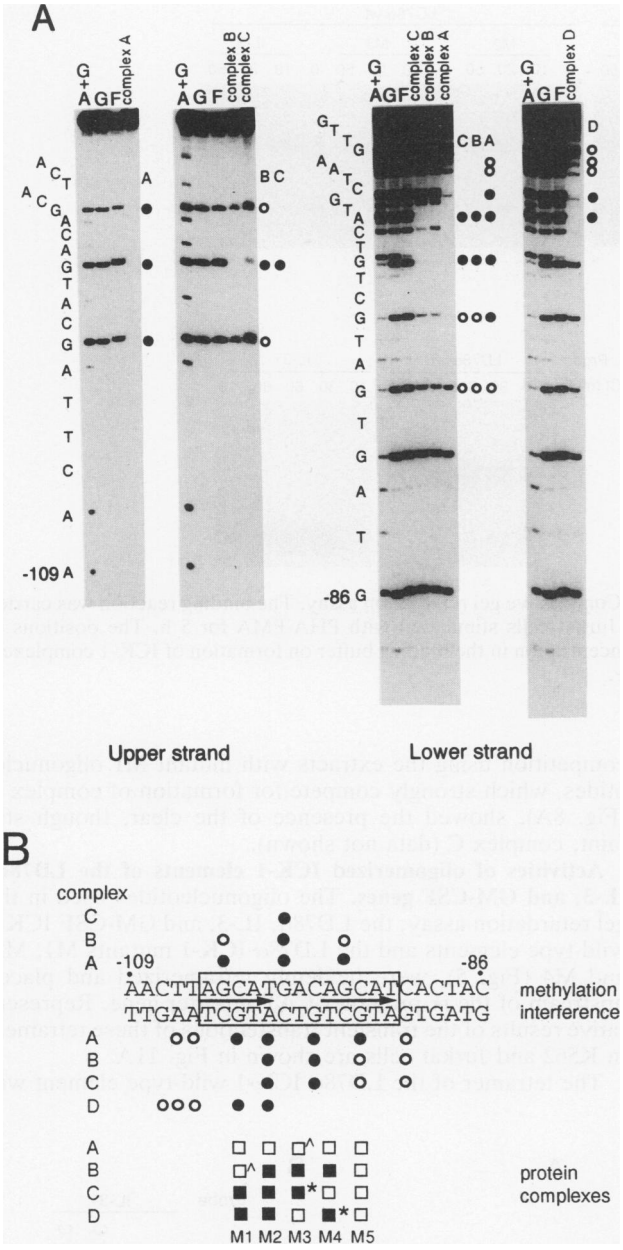

FIG. 7. Methylation interference of LD78α ICK-1 complexes A to D. (A) Methylation interference assay. The binding reaction was carried out at 0°C in the absence or presence of poly(dA-dT) for elution of complex D or other complexes, using extracts prepared from Jurkat cells stimulated with PHA-PMA for 5 h. For analyses of upper and lower strands, the shorter and longer LD78α ICK-1 wild-type oligonucleotides (Fig. 5) were methylated and used in preparative gel retardation. G+A and G show the sequence ladder of each strand, and F indicates unbound probe. The methylated nucleotides which efficiently or partially prevented complex formation are indicated at the right of each panel by filled or open circles, respectively. (B) Positions of nucleotides which interfere with complex formation when methylated. The nucleotide sequence of ICK-1 element is boxed; other symbols are as in panel A. The effects of single-base-pair mutations on the formation of complexes A to D from Fig. 6B are summarized. Open squares, no significant inhibitory effect on complex formation; filled squares, prevention of complex formation; squares with asterisks or care to, partial inhibiting or enhancing effect.

protein. The interference pattern of complex D on the lower strand overlapped those of the other three proteins (strong interference at positions −100 and −102 and partial interference at positions −105, −106, and −107). Although partial interference at positions −91 and −94 was also observed with complex D, this effect is probably due to a minor complex comigrating with complex D.

The nucleotides recognized by the proteins forming each complex are shown in Fig. 7B together with the effects of single-point changes on binding analyzed by gel retardation assay (Fig. 6B). These results are consistent, though there are two exceptions. First, methylation of G's at positions −94 and −102 and A at position −100 on the lower strand strongly interfered with formation of complex A, while transversions at those positions (mutations M1, M2, and M5) had no effect on binding of the ICK-1A protein. Methylated nucleotides might interfere with binding due to structural hindrance. Second, mutation M1 (position −102) prevented formation of complex C, whereas methylation of G on the lower strand at that position had no effect on binding.

The ICK-1A protein exhibits a low binding affinity for the LD78α ICK-1 element. To examine the affinities of protein binding to the LD78α ICK-1 element, competition experiments were carried out. Formation of complexes with LD78α, IL-3, and GM-CSF ICK-1 wild-type probes in extracts from stimulated Jurkat cells was studied by using increasing quantities (10-, 20-, and 50-fold molar excess) of wild-type and mutant oligonucleotides at 0°C in buffer containing poly(dA-dT) and 60 mM KCl.

When LD78α wild-type oligonucleotides were used as probes (Fig. 8A), mutant M1 competed more efficiently for formation of complex B than did the wild type and was unable to competitively inhibit the formation of complex C. Mutant M4 had no or little effect on the formation of complex B and competitively inhibited the formation of complex C to the same degree as did the wild type. Mutations M2 and M3 also had no or little effect on the formation of complexes B and C. These results were consistent with those shown in Fig. 6B. However, both the LD78α wild type and the mutants were unable to competitively inhibit the formation of complex A, but a higher concentration (200-fold molar excess) of the LD78α ICK-1 wild-type competitor reduced the formation of complex A (data not shown). In contrast, the formation of complexes A and C was completely inhibited by the IL-3 ICK-1 oligonucleotides. When IL-3 ICK-1 oligonucleotides were used as probes, GM-CSF ICK-1 oligonucleotides competed efficiently for formation of complex A (Fig. 8B). The same result was obtained when the GM-CSF and IL-3 ICK-1 oligonucleotides were used as the probe and competitor, respectively.

These results suggest that the ICK-1A protein has a lower binding affinity for the LD78α ICK-1 element than do the ICK-1B and -C proteins and a higher binding affinity for the IL-3 ICK-1 element than for the LD78α ICK-1 element. It is interesting to note that when LD78α and IL-3 ICK-1 oligonucleotides were used as the probe and competitor, respectively, a complex just above complex C was increased while the formation of complexes A and B was reduced. This result suggests that the protein forming this LD78α-specific complex exhibits a lower affinity than does the ICK-1A or -C protein.

These results were also supported by the salt concentration dependency of the interaction of each factor with the element (Fig. 8C). When the binding reaction was carried out at various KCl concentrations with the LD78α wild-type probe, ICK-1A, -B, and -C proteins bound efficiently at 10,

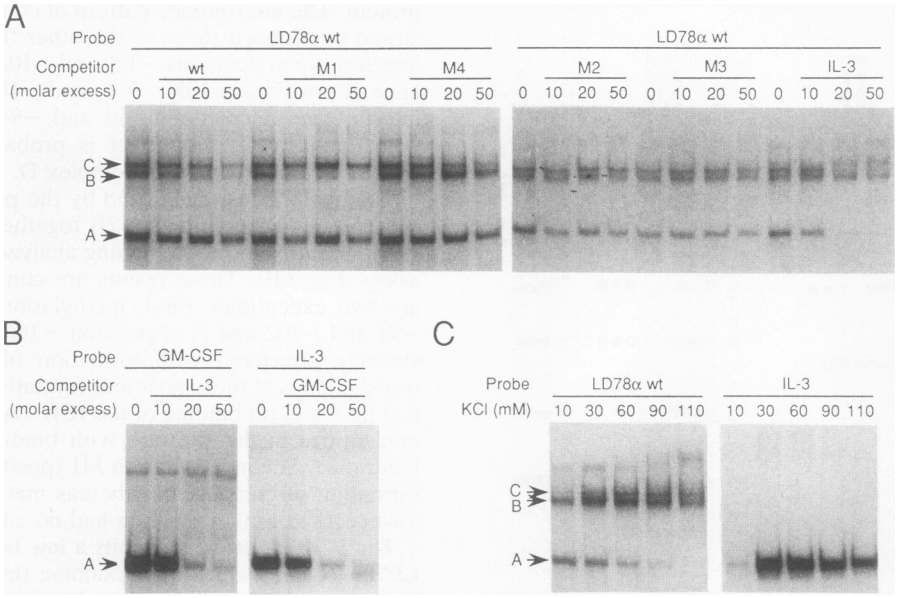

FIG. 8. Relative affinities of LD78α ICK-1 binding proteins. (A and B) Competitive gel retardation assay. The binding reaction was carried out at 0°C in the presence of poly(dA-dT) with extracts prepared from Jurkat cells stimulated with PHA-PMA for 5 h. The positions of complexes A, B, and C are indicated by the arrows. (C) Effects of KCl concentration in the binding buffer on formation of ICK-1 complexes. The binding reaction was carried out as in panels A and B. wt, wild type.

30, and 60 to 90 mM KCl, respectively. However, the IL-3 ICK-1 probe bound ICK-1A protein most efficiently at 30 mM KCl, and the binding efficiency was very low at 10 mM KCl. These results clearly indicate that the ICK-1B and -C proteins have higher binding affinities for the LD78α ICK-1 element than does the ICK-1A protein at around 60 mM KCl. Similar results were obtained with K562 extracts (data not shown).

Temperature and salt concentration affect binding of the ICK-1A protein. As shown in Fig. 6A, the formation of complex A was reduced when the binding reaction was carried out at 30°C in buffer containing 60 mM KCl. We further investigated the effect of incubation temperature on the formation of complex A at a lower KCl concentration, using the IL-3 ICK-1 probe. Contrary to observations at 60 mM KCl, formation of complex A was increased when the binding reaction was carried out at 30°C in buffer containing 10 mM KCl (Fig. 9A).

The formation of IL-3 complex A was shown to be reversible (Fig. 9B). When the binding reaction was carried out at 0°C for 30 min in buffer containing 60 mM KCl and the mixture was then incubated at 30°C for 30 min, complex A formation decreased to 21% of the value observed after incubation at 0°C for 1 h. However, when the incubation temperature was shifted from 30 to 0°C, complex A formation was 42% of that seen after incubation at 0°C for 1 h. These results suggest that incubation at 30°C results in dissociation of the DNA–ICK-1A complex, with partial reassociation after incubation at 0°C. It is interesting to note that incubation in 60 mM KCl at 30°C for 1 h (Fig. 9B) rather than 30 min (Fig. 9B and 6A) resulted in complete disappearance of complex A.

ICK-1-binding proteins are constitutively expressed in K562 and Jurkat cells. All of the ICK-1 complexes were formed in the extracts prepared from K562 and Jurkat cells before and after stimulation (Fig. 10). Complex C formed with extracts from unstimulated Jurkat cells was very faint. However, competition using the extracts with mutant M1 oligonucleotides, which strongly compete for formation of complex B (Fig. 8A), showed the presence of the clear, though still faint, complex C (data not shown).

Activities of oligomerized ICK-1 elements of the LD78α, IL-3, and GM-CSF genes. The oligonucleotides used in the gel retardation assay, the LD78α, IL-3, and GM-CSF ICK-1 wild-type elements and the LD78α ICK-1 mutants M1, M2, and M4 (Fig. 5), were tandemly tetramerized and placed upstream of the *tk* promoter-CAT reporter gene. Representative results of the transient transfections of these tetramers in K562 and Jurkat cells are shown in Fig. 11A.

The tetramer of the LD78α ICK-1 wild-type element was

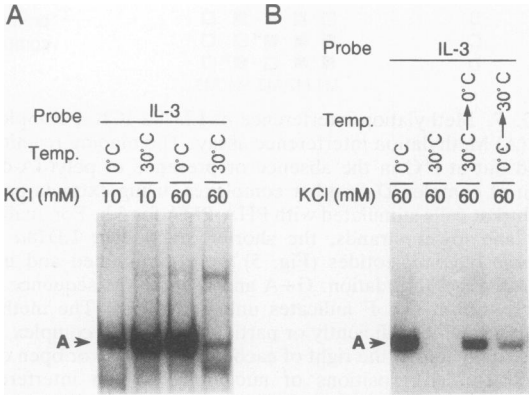

FIG. 9. Effects of incubation temperature and KCl concentration in the binding buffer on formation of IL-3 ICK-1 complex A. Binding was carried out in buffer containing poly(dA-dT) and KCl at the concentration indicated. Incubation was at 0 or 30°C for 30 min (A) or 1 h (B), except in the two rightmost lanes in panel B, in which case incubation was carried out at 30°C for 30 min followed by 30 min at 0°C or vice versa as indicated.

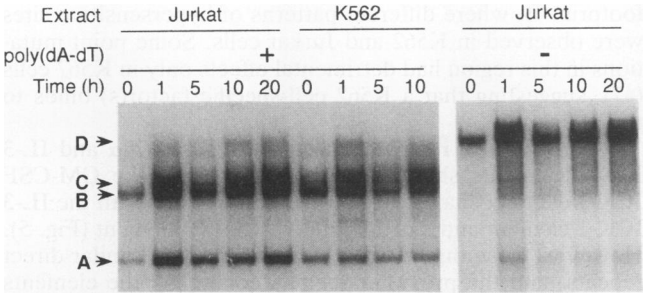

FIG. 10. Time course of LD78α ICK-1 protein binding. Nuclear extracts were prepared from cells unstimulated (0 h) or stimulated with PMA (K562) or PHA-PMA (Jurkat) for 1 to 20 h as indicated. Binding was carried out at 0°C in the absence (−) or presence (+) of poly(dA-dT).

inactive in both cell types in the absence or presence of stimulation. Because the ICK-1 element is bound by several proteins, including negative and positive factors, this result suggests that if at least one of the four binding sites was occupied by the negative factor, this factor could repress the function of positive factors bound to other sites on the same plasmid. The tetramers of LD78α ICK-1 mutants M2 and M4 were also inactive. However, the tetramer of mutant M1 exhibited significant activity in both cell types even in the absence of stimulation. This finding is consistent with the observation that the ICK-1B positive factor was bound more efficiently by this mutant than by the wild-type sequence (Fig. 6B and 8A). This mutant is also recognized by the ICK-1A protein, which is probably a negative factor, but a substantial population of the M2 tetramers may have been bound only by the positive factors in vivo. However, the increase in activity of this construct, approximately two- and sevenfold in K562 and Jurkat cells, respectively, upon stimulation, was unexpected because the ICK-1B factor is constitutive (Fig. 10). It may be that this factor is modified on stimulation resulting in more effective stimulation of transcription. The tetramer of the IL-3 ICK-1 element, like that of the LD78α ICK-1 element, had no activity. Surprisingly, the tetramer of the GM-CSF ICK-1 element had considerable activity in the absence of stimulation. Upon stimulation, this activity was increased about 3-fold in K562 cells and 17-fold in Jurkat cells. Although ICK-1A binds to this element with a high affinity (Fig. 8B), the high activity of the tetramer suggests that ICK-1A could not completely repress the activity of inducible GM-CSF ICK-1-specific positive factors bound to sites other than those occupied by the ICK-1A negative factor.

To determine whether the negative factor can repress the activities of positive factors other than those bound to ICK-1 sites, the tetramers were also placed in front of the *tk* promoter-CAT gene of pBL2SVE (50), which contains the simian virus 40 (SV40) enhancer inserted 3′ to the CAT gene. Since the SV40 enhancer was not active in unstimulated cells, only the result in stimulated cells is shown (Fig. 11B). Transfection was carried out by using Jurkat cells with an RSV-luciferase expression plasmid as an internal control.

The LD78α wild-type and M2 and the IL-3 ICK-1 elements reduced the SV40 enhancer activity in stimulated Jurkat cells to 65, 63, and 29%, respectively, of the level of the pBL2SVE (100%). However, the LD78α M1 and GM-CSF ICK-1 elements appeared to have no or little effect on the enhancer activity. These results support the results from

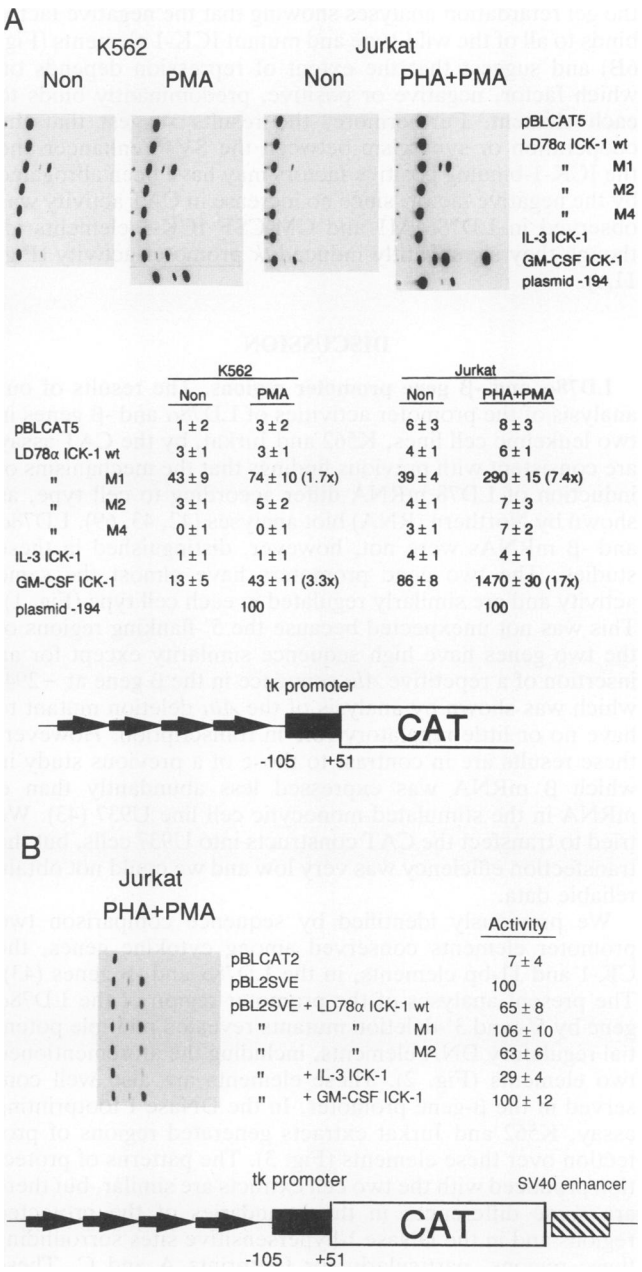

FIG. 11. Activities of tandem repeats of ICK-1 elements. The nucleotide sequence of one unit constituting tandemly tetramerized ICK-1 repeats is shown in Fig. 5. Relative CAT activities of the tetramers placed in front of the *tk* promoter of pBLCAT5 (A) or pBL2SVE (B) are shown, and the activity of each construct is the mean of three experiments ± standard error. Activities shown in panel B are normalized against the level of luciferase expression. In panel A, the activity of the 5′-promoter deletion mutant, plasmid −194, transfected as a control in the presence of stimulation in each cell type is taken as 100%. The increase in induction of tetramers, LD78α ICK-1 M1 and GM-CSF ICK-1, is shown in parentheses. In panel B, the activity of the pBL2SVE in the presence of stimulation is taken as 100%. Construction of the plasmids containing four ICK-1 repeats is schematically represented. pBL2SVE (50) is the derivative of pBLCAT2 containing the SV40 enhancer downstream of the CAT gene. pBLCAT2 is identical to pBLCAT5 except that pBLCAT5 contains one more SV40 poly(A) signal sequence in front of multiple cloning sites (33). Each arrow represents one unit of ICK-1 repeats. wt, wild type.

the gel retardation analyses showing that the negative factor binds to all of the wild-type and mutant ICK-1 elements (Fig. 6B) and suggest that the extent of repression depends on which factor, negative or positive, predominantly binds to each element. Furthermore, the results suggest that the cooperation or synergism between the SV40 enhancer and the ICK-1-binding positive factors may have been abrogated by the negative factor, since no increase in CAT activity was observed in LD78α M1 and GM-CSF ICK-1 elements although they significantly induced *tk* promoter activity (Fig. 11A).

DISCUSSION

LD78α and -β gene promoter regions. The results of our analysis of the promoter activities of LD78α and -β genes in two leukemic cell lines, K562 and Jurkat, by the CAT assay are consistent with previous findings that the mechanisms of induction of LD78 mRNA differ according to cell type, as shown by Northern (RNA) blot analyses (42, 43, 69). LD78α and -β mRNAs were not, however, distinguished in these studies. The two gene promoters have almost the same activity and are similarly regulated in each cell type (Fig. 1). This was not unexpected because the 5′-flanking regions of the two genes have high sequence similarity except for an insertion of a repetitive *Alu* sequence in the β gene at −294, which was shown by analysis of the *Alu* deletion mutant to have no or little regulatory role in transcription. However, these results are in contrast to those of a previous study in which β mRNA was expressed less abundantly than α mRNA in the stimulated monocytic cell line U937 (43). We tried to transfect the CAT constructs into U937 cells, but the transfection efficiency was very low and we could not obtain reliable data.

We previously identified by sequence comparison two promoter elements conserved among cytokine genes, the CK-1 and 11-bp elements, in the LD78α and -β genes (43). The present analyses of the promoter region of the LD78α gene by 5′- and 3′-deletion mutants revealed multiple potential regulatory DNA elements, including the aforementioned two elements (Fig. 2). These elements are also well conserved in the β-gene promoter. In the DNase I footprinting assay, K562 and Jurkat extracts generated regions of protection over these elements (Fig. 3). The patterns of protection produced with the two cell extracts are similar, but there are some differences in the boundaries of the protected regions and in the DNase I-hypersensitive sites surrounding these regions, particularly for footprints A and C. These protected regions produced in K562 and Jurkat extracts are slightly different, and K562 extracts generate hypersensitive sites which are not evident with Jurkat extracts. In particular, the hypersensitive site associated with K562 footprint A appeared in extracts prepared from stimulated cells. Furthermore, protection of the footprint A region by extracts from unstimulated cells is much less pronounced than that by extracts from stimulated cells. These results strongly suggest that these regions are recognized by cell- and/or stimulation-specific transcriptional factors that allow transcription of LD78 genes in a cell-type-specific manner upon stimulation. However, protection by cell-specific or stimulation-specific factors may have been obscured by constitutive or ubiquitous factors which bind to the overlapping regions. Therefore, no clear difference was observed between the regions protected from the unstimulated and stimulated extracts. Another candidate for the binding site of a cell-specific factor is the region immediately upstream of footprint B, where different patterns of hypersensitive sites were observed in K562 and Jurkat cells. Some point mutations in this region had detrimental effects only in K562 cells (47), suggesting that a K562 cell-specific factor(s) binds to the region.

ICK-1 negative regulatory element. The LD78α and IL-3 ICK-1 elements share 11 of 14 nucleotides; the GM-CSF ICK-1 element shares only 7 of 14 nucleotides with the IL-3 ICK-1 element and 8 of 14 with the LD78α element (Fig. 5). However, these three ICK-1 elements contain similar direct repeats, and the promoter regions containing the elements have been shown to have transcriptional inhibitory activity by analyses of deletions and point mutations (37, 45) (Fig. 2B). Promoter activities of single-point mutations in the LD78α ICK-1 element suggested interaction of positive and negative transcriptional factors with this element (Fig. 4), and similar observations have been reported with the GM-CSF ICK-1 element (41). Consistent with these observations, Jurkat and K562 nuclear extracts prepared from both unstimulated and stimulated cells formed more than two DNA-protein complexes with these ICK-1 elements (Fig. 6A). One complex, ICK-1 complex A, with the same mobility and with the same sensitivity to high incubation temperature in buffer containing 60 mM KCl, was observed with all three ICK-1 elements, suggesting that the protein forming this complex, ICK-1A, is the negative factor that binds to the direct repeat found in the negative regulatory elements of the three cytokine genes. Supporting this notion, the strong contact sites required for the binding were found within the repeats by methylation interference analysis (Fig. 7). These sites are identical with those reported for the IL-3 ICK-1 negative factor (NIP) (37). ICK-1A is constitutively expressed in both K562 and Jurkat cells (Fig. 10) and is involved in repression of LD78α gene transcription. The LD78α ICK-1 element is active even in the absence of stimulation, as shown by the weak promoter activity in the absence of stimulation of the 3′-promoter deletion mutant, plasmid −95, which lacks 3 bp of the 3′-end of the ICK-1 element. This 3-bp deleted sequence includes one of the strong contact sites (at position −94) of ICK-1A, but the sequence is not required for efficient binding of the other ICK-1 proteins. In contrast, none of the single-base-pair mutants increased the basal promoter activity in the absence of stimulation in vivo (Fig. 4), and none of the corresponding point mutations in the LD78α ICK-1 element significantly reduced the formation of complex A in vitro (Fig. 6B). Therefore, the negative factor ICK-1A may consist of two subunits, each recognizing the half repeat. Binding of ICK-1A to these point mutants may still occur if the other half is intact.

The recognition sequence of ICK-1A overlaps those of the ICK-1B, -C, and -D proteins. ICK-1A binds with a lower affinity than do the ICK-1B and -C proteins (Fig. 8). It also appears to have a lower affinity than does the ICK-1D protein, because when the ICK-1D protein was abolished by poly(dA-dT), ICK-1A bound efficiently to mutant M3, which was not an efficient competitor for the ICK-1A–LD78α ICK-1 wild-type complex. The binding affinity of ICK-1A was strongly influenced by incubation temperature and salt concentration (Fig. 9). Low temperature (0°C) and high KCl concentration (60 mM) or high temperature (30°C) and low KCl concentration (10 mM) may stabilize the interaction between ICK-1A and the ICK-1 element or between the subunits of ICK-1A. This stabilization results in an increased binding affinity of the factor for the element, the temperature shift experiment suggesting that this effect is reversible.

However, the binding conditions still seem to be different from the physiological conditions. There may be a labile stabilizing factor that was not detected in vitro.

The gel retardation patterns for Jurkat extracts with the GM-CSF ICK-1 element shown here are slightly different from those reported by Miyatake et al. (41). In their study, the binding reaction was carried out at 30°C in buffer containing 98 mM KCl under conditions in which ICK-1 complex A is barely detectable. They also analyzed the effects of point mutations in the GM-CSF ICK-1 element in in vitro transcription experiments in which incubation was carried at 30°C in 60 mM KCl. Although the same mutations upregulated promoter activities in vivo, the authors did not observe upregulation in vitro. In our DNase I footprinting experiments, the binding reaction was carried out at 0°C in buffer containing 30 mM KCl and subsequent DNase I digestion was done at 30°C for 1 min. However, in the control gel retardation analyses, the brief exposure of the reaction mixture to 30°C for 1 min after incubation at 0°C for 30 min had no effect on the formation of LD78α complex A (data not shown).

The constitutively expressed protein, ICK-1B, was shown to be a positive regulatory factor. The factor was bound efficiently by the single-base-pair mutant M1, and the corresponding mutation in the LD78α promoter upregulated promoter activity. The M1 oligonucleotide tetramer was active in the absence of stimulation, and the activity was enhanced upon stimulation, particularly in Jurkat cells, probably by activation of the factor by modification. Involvement of the ICK-1C protein, which is present in small quantities in unstimulated Jurkat cells but at increased levels upon stimulation, in the induction of the M1 tetramer can be ruled out because formation of complex C was not observed with mutant M1 oligonucleotides. Although the functions of the ICK-1C and -D proteins have not been clearly determined in this study, ICK-1C is clearly not important for IL-3 gene expression even if it does function as a positive factor. Removal of the entire IL-3 ICK-1 element by 5′-promoter deletion did not result in down-regulation of the promoter activity (37).

The ICK-1B and -C proteins recognize almost the same nucleotides, i.e., the central region of the LD78α element. However, only the ICK-1A and -C proteins bind to both the LD78α and IL-3 ICK-1 elements, the ICK-1B protein not binding to the IL-3 ICK-1 element. The central regions of both elements are identical except for one nucleotide, A at position −98 in the LD78α element and G at the corresponding position in the IL-3 element. Therefore, G at that position may prevent the binding of ICK-1B but not ICK-1C, although the transversion A to C at the same position in the LD78α ICK-1 element inhibited binding of both proteins. The nucleotide difference at that position and/or those at the 3′ end of the element, T and C at positions −92 and −91 in the LD78α element and two G's at the corresponding sites in the IL-3 element, may account for the higher binding affinity of ICK-1A for the IL-3 ICK-1 element than for the LD78α ICK-1 element. However, the direct repeats of IL-3 and GM-CSF ICK-1 elements, to which ICK-1A binds with almost the same affinity, are slightly different. Further study on the best sequence for recognition must await the purification of ICK-1A so that its binding can be investigated without influence from other binding proteins.

The recognition sequence of the ICK-1D protein was shown to lie in the 5′-border region of the ICK-1 element. This sequence is relatively AT rich, and this may be the reason why formation of complex D was inhibited by poly(dA-dT). IL-3 and GM-CSF ICK-1 elements also formed poly(dA-dT)-sensitive complexes. The central region of the GM-CSF ICK-1 element includes the AT-rich inverted repeat ATTAAT. This sequence may therefore be important for binding of the proteins forming slowly migrating poly(dA-dT)-sensitive complexes. These proteins may correspond to the positive factors mediating induction of the GM-CSF promoter (41, 44).

Promoter activities of the site-directed mutants M1 and M4 in stimulated K562 cells were lower than that of the wild type, in contrast to their activities in stimulated Jurkat cells. Although no apparent difference was observed between K562 and Jurkat extracts in formation of complexes with the LD78α ICK-1 element, the region immediately upstream of the element was shown to be important for induction of the LD78 promoter in K562 cells by the 5′-promoter deletion study (Fig. 2A) and analyses of point mutations in the region (47). Therefore, mutants M1 and M4 may affect the binding of the positive factor(s) to that region directly or by a DNA conformational change. Alternatively, the mutants may abolish the interaction or cooperation of the factor(s) with other positive factors bound by each mutant.

It seems likely that the ICK-1A negative factor represses transcription by two different mechanisms. One mechanism is competition between ICK-1A and the positive factor(s) at the single ICK-1 element. In the LD78α promoter, it is likely that ICK-1A competitively inhibits binding of the ICK-1B positive factor, which is important for LD78α gene transcription. Therefore, if ICK-1A binds to the LD78α ICK-1 element with high affinity, then LD78α promoter activity will be completely lost. The same may be true for the GM-CSF ICK-1 element. Although ICK-1A binds to this element with high affinity, the positive factors important for GM-CSF gene expression may have higher binding affinities. Thus, the ICK-1A negative factor acts as a modulator in cytokine gene transcription, regulating the activity of the positive factor(s) bound to the same site. However, the activities of tetramers of ICK-1 elements suggested a second possible mechanism, that ICK-1A partially represses the activities of positive factors bound to different sites (Fig. 11). The negative factor in particular may work mainly by this mechanism in the IL-3 promoter, because no important positive factor binds to the IL-3 ICK-1 element and its tetramer inhibits the SV40 enhancer more effectively than do the other two cytokine ICK-1 elements. Therefore, in the context of natural promoters in which each single ICK-1 element of three cytokine genes is surrounded by different positive regulatory elements, the functions of the positive factors bound to those sites may be affected by the negative factor, the extent of the repression possibly depending on the nature of the positive regulatory factors. Purification and subsequent characterization of ICK-1A will elucidate more clearly the mechanisms of repression.

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