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Positive and negative elements regulate human interleukin 3 expression

(gene regulation/promoter function/AP-1 complex/T-cell activation)

BERNARD MATHEY-PREVOT*, NANCY C. ANDREWS, HEATHER S. MURPHY, SUSAN G. KREISSMAN, AND DAVID G. NATHAN

Division of Hematology Oncology, The Children's Hospital; Department of Pediatric Oncology, The Dana–Farber Cancer Institute; and Department of Pediatrics, Harvard Medical School, Boston MA 02115

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ABSTRACT **The human interleukin 3 (IL-3) promoter is comprised of several cis-acting DNA sequences that modulate T-cell expression of IL-3. These are located within 315 nucleotides upstream of the mRNA start site. Transient expression of reporter genes linked to serially deleted sequences of the IL-3 promoter has allowed mapping of two activator sequences and an interposed repressor sequence. The proximal regulatory region is specific to IL-3 and prerequisite for efficient transcription. Its effect is enhanced by a second, more distal activating sequence consisting of an AP-1 binding site. Between the two activators lies a transcriptional silencer, which is a potent repressor in the absence of the AP-1 site. DNA–nuclear protein binding experiments demonstrate specific complex formation within each of these functional regions. Thus, both positive and negative regulatory elements appear to control expression of the human IL-3 gene in activated T cells.**

Interleukin 3 (IL-3) is a key regulatory molecule in the early differentiation of hematopoietic precursor cells (1). It is produced by activated T lymphocytes and natural killer cells (2, 3) and appears in these cells to be coregulated with granulocyte–macrophage colony-stimulating factor (GM-CSF), which is structurally and functionally related but nonhomologous (4). Interestingly, the genes for both cytokines are adjacent to each other on human chromosome 5 (5), nested within a cluster of genes for growth factors and growth factor receptors. In addition, IL-3 and GM-CSF share conserved elements in their promoter sequence (6, 7), suggesting common regulatory mechanisms in their expression. However, in contrast to IL-3, GM-CSF expression is readily detected in activated macrophages, fibroblasts, and endothelial cells (4). To understand the basis for the apparent T-cell-specific expression of IL-3 and what distinguishes IL-3 regulation from that of GM-CSF, we undertook the characterization of IL-3 upstream regulatory sequences.

T-cell activation results in rapid expression of the IL-3 gene and requires signaling through the bindings of an antigen to the T-cell receptor complex and of interleukin 1 (IL-1) released by the antigen-presenting cell to its receptor (8). The ensuing calcium flux and protein kinase C activation (9) trigger a series of biochemical events that lead to the transcriptional activation of several genes, including IL-3. In this report, we show that the target DNA sequences for IL-3 regulation are contained within a 315-nucleotide (nt) region upstream of the cap site. Although present within this fragment, the conserved elements CK-1 and CK-2 of GM-CSF and IL-3 promoters (7) do not appear to play a role in T-cell expression of IL-3. Instead, expression in activated T cells results from the combined presence of an AP-1 site (10) and a specific activating region.

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MATERIALS AND METHODS

Cells. MLA 144 cells (11) were grown at 37°C, 5% CO_2 in RPMI 1640 complete medium (which contains 10% fetal calf serum, penicillin/streptomycin, and 50 μM 2-mercaptoethanol).

DEAE-Dextran Transfection and RNA Isolation. Transfection was performed by the DEAE-dextran technique. Cells (4×10^7) were sequentially washed with cold serum-free RPMI 1640 medium and cold TBS (25 mM Tris·HCl, pH 7.4/137 mM NaCl/5 mM KCl/0.7 mM $CaCl_2$/0.5 mM $MgCl_2$/0.6 mM $NaPO_4$, pH 7.4). Cells were then resuspended in 4 ml of a TBS solution containing DEAE-dextran (0.25 mg/ml), DNA (30 μg), and chloroquine (0.1 μM) and incubated at 37°C in 5% CO_2 for 30 min. Cells were washed and resuspended at 0.5×10^6 per ml in RPMI 1640 complete medium and further incubated for 36 hr. At that time, phorbol 12-myristate 13-acetate (PMA) was added (10 ng/ml). After 9-hr incubation, cells were lysed, and RNA was isolated (2).

RNase Protection Assay. Twenty-five μg of total RNA was resuspended in 31 μl of hyb-buffer [80% (vol/vol) formamide/40 mM Pipes, pH 6.4/400 mM NaCl/1 mM EDTA] containing the appropriate ^{32}P-labeled RNA probe. RNase protection was as described (12). Hybridization was done at 52°C overnight; digestion with RNase A and T_1 was at 42°C for 30 min. Reactions were treated with proteinase K at 37°C for 30 min, extracted with phenol/chloroform, and loaded on 6% Tris borate EDTA (TBE) acrylamide/urea gel.

For probe, a *Sma* I–*Pst* I fragment containing exon 1 of the IL-3 gene was modified by insertion of a *Xho* I linker (12-mer) at a unique *Hpa* I site and subcloned into SPT18 (Boehringer Mannheim). The antisense probe (330 nt) is obtained with SP6 polymerase after linearization with *Eco*RI (Fig. 1).

IL-3 Reporter Genes. The reporter genes consist of human genomic IL-3 sequences with various lengths of upstream sequences and terminate at an *Eco*RI site downstream of the last exon (5). The first exon carries a 12-mer *Xho* I linker insertion at a unique *Hpa* I site. The following restriction sites were used to generate the constructs: *Stu* I, −315; *Eco*NI, −283; *Eco*0109I, −271; *Sca* I, −173; *Fok* I; −143; *Aha* II, −97; *Sma* I, −61. A unique *Sph* I site was created at −250 by site-directed mutagenesis. Internal deletions within the −315 promoter were obtained by using the *Sca* I and *Sma* I sites; for ΔCKs, *Nla* IV (within CK-1) and *Aha* II sites were used. Oligonucleotides that contained the nuclear inhibitory pro-

Abbreviations: IL-1, -2, and -3, interleukin 1, 2, and 3, respectively; GM-CSF, granulocyte–macrophage colony-stimulating factor; nt, nucleotide(s); PMA, phorbol 12-myristate 13-acetate; CRE, cAMP-responsive element.

*To whom reprint requests should be addressed.

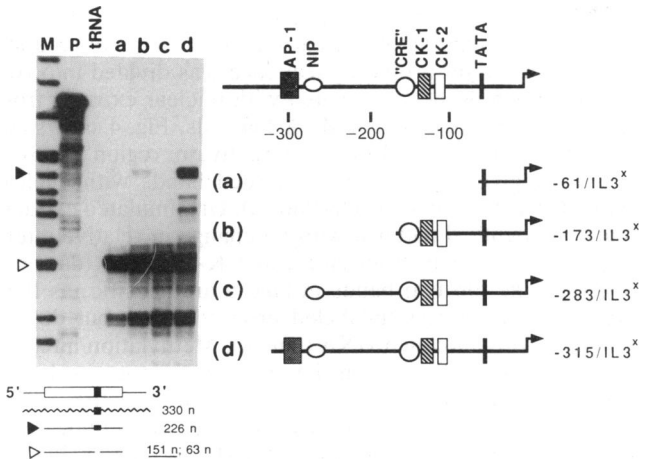

FIG. 1. Mapping of the IL-3 promoter by an expression assay in MLA 144 cells. MLA 144 cells were transfected with 30 μg of purified plasmid DNA, grown for 36 hr, and stimulated for 9 hr with PMA (10 ng/ml). Constructs [(a)–(d)] are diagramed next to their respective lanes (a–d). Transfections were repeated at least three times, often with a different preparation of a given plasmid, with identical results. Twenty-five μg of total RNA was hybridized to the modified IL-3 first-exon RNA probe. After digestion with RNase A and T_1, reaction products were separated on 6% sequencing gel. Potential regulatory sites included in each reporter gene are indicated. P, untreated probe; M, DNA markers (*Msp* I digest of pBR322). Closed and open arrowheads indicate the 226-nt and the 151-nt fragments protected by the reporter gene and gibbon endogenous transcripts, respectively. The bottom third of the gel, which contains the 63-nt protected band, is not shown.

tein (NIP) sequence (5′-GATCCTCTCACCTGCTGCCAT-GCTTCCCAT-3′; 5′-CTAGATGGGAAGCATGGCAG-CAGGTGAGAG-3′) were synthesized, hybridized, and ligated upstream of construct −173/IL3^x to generate (NIP)–173/IL3^x.

Protruding ends generated after digestion with restriction enzymes were blunted with DNase I large-fragment polymerase. Details pertaining to the derivation of the constructs are available upon request.

DNA-Binding Assays, DNase Footprinting and Methylation Interference. Preparation of nuclear salt-wash extracts from MLA 144 cells was according to Dignam *et al.* (13). Gel shift, DNase I footprinting, and methylation interference were done as described (14). Gel-shift studies were performed in 15-μl reaction containing 1 μl (≈5 μg) of crude nuclear extract and 2.5 μg of poly(dI·dC) in buffer B [10 mM Hepes, pH 7.8/50 mM potassium glutamate/5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/5% (vol/vol) glycerol]. For competition experiments, excess unlabeled competitors were preincubated with the crude extract at 0°C for 15 min before the probe was added. DNA–protein complexes were separated on nondenaturing acrylamide gels (6.5%). DNase I protection was done in 50-μl reactions containing 0.5 ng of end-labeled DNA, 2.5 μg of poly(dI·dC), and 50 μg of crude nuclear extract in buffer B with 1 mM EDTA and 2% (vol/vol) polyvinyl alcohol. After phenol/chloroform extraction, samples were resuspended in formamide/dyes solution and electrophoresed through 8% TBE/urea sequencing gels. Methylation interference experiments were done as described (14).

RESULTS

To identify cis-acting DNA sequences responsible for IL-3 expression in activated T cells, we selected a particular T-cell line, MLA 144, derived from an ape infected with gibbon leukemia virus (11). These cells have the advantageous property that phorbol 12-myristate 13-acetate (PMA) treatment will trigger high expression of IL-3 and GM-CSF with identical kinetics to those found in normal T cells after PMA/phytohemagglutinin activation (2). Moreover, run-on experiments performed with MLA 144 cells show clear transcriptional activation of IL-3 and GM-CSF genes after 3- or 9-hr stimulation with PMA (data not shown).

The sequence extending from the second exon to ≈685 nt upstream of the putative start site of the IL-3 gene was determined. It was similar to the published sequence (6), except for a T → C change already reported (3), resulting in a proline residue at amino acid 27. Sequence comparison revealed several potential regulatory sites in the first 315 nt of the IL-3 promoter (Fig. 1). A TATA box was found at position −30; starting at nt −111 we identified CK-2 and CK-1, two elements conserved in the promoter of several cytokines (7). Upstream of CK-1, a run of 8 nt was observed to be homologous to the cAMP-responsive element (CRE) and transcription factor ATF-binding site (15), with 6 of 7 nt matching the consensus sequence, both on the coding and noncoding strands. Finally, there was an AP-1-binding site at nt −296.

To determine whether these motifs could regulate IL-3 expression, we transfected into MLA 144 cells reporter genes linked to various lengths of the 315-nt upstream region. To mimic the physiologic system as closely as possible, we used the genomic IL-3 gene as a reporter gene. To discriminate its expression from that of the endogenous gibbon IL-3 gene, we introduced a 12-base-pair insertion at the *Hpa* I site in the first exon of the reporter genes. When a probe complementary to this modified first exon is used, transcripts derived from the reporter genes give rise, in an RNase protection assay, to a 226-nt protected fragment (Fig. 1). On the other hand, transcripts from gibbon IL-3 gene, which lack the 12-base-pair insertion, give rise to two protected fragments of 151 and 63 nt.

Four constructs with increasing lengths of the IL-3 promoter were initially tested (Fig. 1). Only results with stimulated MLA 144 cells are presented, as none of the constructs are expressed in the absence of PMA treatment. The presence of a TATA box is not sufficient for IL-3 expression (lane a). In contrast, the first 173 nt of the promoter can mediate expression of the IL-3 gene (lane b). Surprisingly, the inclusion of another 110 upstream nt causes a drastic loss of expression (lane c), suggesting the presence of inhibitory sequences between nt −173 and −283. Adding 31 nt past this putative repressor site not only abolishes the inhibition of expression but further enhances its overall level (lane d). This second activator site coincides with the AP-1 site.

As several candidate regulatory sites had been recognized within the first 173 nt, this region was dissected further (Fig. 2). Lanes a and d demonstrate again that there is an activator region between nt −61 and −173. Inclusion of promoter sequence to nt −97 does not promote any significant expression. Similarly, only weak expression is seen with the first 143 nt of the IL-3 promoter, despite the presence of CK-1 and CK-2 motifs (lane c). Thus, if CK-1 and CK-2 play a role in IL-3 expression, they are clearly not sufficient. Interestingly, promoter sequences in construct −143/IL3^x stop 2 nt short of including the full CRE/ATF-like element. In preliminary experiments, the full CRE/ATF-like site was restored, and although a detectable level of expression was seen with this construct, it was nonetheless lower than the level obtained with construct −173/IL3^x (data not shown). Therefore, nucleotides upstream of the CRE/ATF-like site are probably required for the full expression mediated by the −61 to −173 region. This led us to reinvestigate the nature of these nucleotides. Interestingly, immediately upstream of the CRE/ATF-like element are 8 nt (ATGAATAA) that are highly conserved with eight of the bases of the interleukin 2

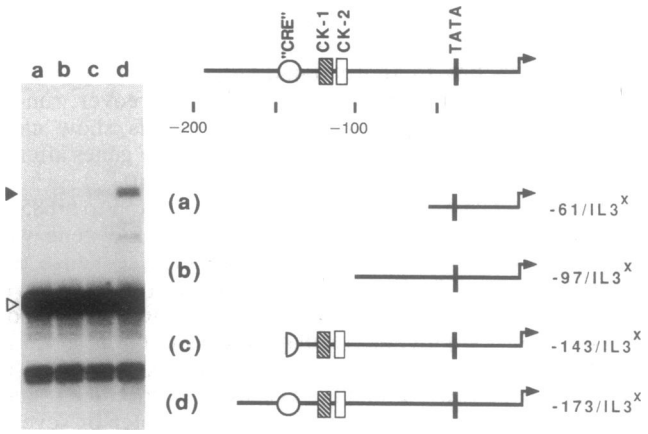

FIG. 2. Deletion analysis of activating region 1. The promoter region for each construct is indicated at right. Transfections, RNase protection, and symbols are described in the legend for Fig. 1.

(IL-2) promoter located between nt −240 and −250 (ATGAATTA) (16). This site in the IL-2 promoter binds the OCT-1 protein (17).

To further evaluate the roles in IL-3 expression of the AP-1, CK-1, and CK-2 regions, other constructs were obtained and their expression was compared to that of construct −315/IL3^x or −173/IL3^x (Fig. 3). Deletion of activator region 1 from the −315 promoter results in a near complete loss of expression (lane b). Thus, the presence of an AP-1 site is not sufficient for IL-3 transcriptional activation; its role, rather, seems to enhance expression mediated by the first activating region. Deletion of most of CK-1 and of the entire CK-2 elements had little effect on expression (lane d), suggesting that they are dispensable for transient IL-3 expression in T cells. Finally, the noted inhibitory region between −173 and −283 was more precisely mapped. As DNA-binding experiments (see below) showed a strong interaction between nt −265 and −255 and a nuclear factor, two new constructs were obtained. One, construct −250/IL3^x, lacked this DNA-binding region and the second, construct −271/IL3^x, just included it. As shown in lane e, expression of construct −250/IL3^x is similar to that of construct −173/IL3^x (lane c). Expression of construct −271/IL3^x, on the other hand, was repressed (lane f). Therefore, the inhibitory region functionally maps to nt −271 to −250. To confirm this result, a synthetic double-stranded oligonucleotide spanning this region was placed upstream of construct −173/IL3^x. Expression of this construct was completely extinguished (lane g).

We next attempted to correlate the above regulatory sequences with binding of nuclear factors by a gel-retardation assay. The 315-nt promoter sequence was divided into two probes. Each probe was treated with nuclear extracts from stimulated or unstimulated MLA 144 cells. Fig. 4 shows our finding with a DNA probe covering activator region 1 (−61 to −173). It gives rise to three retarded bands with nuclear extracts from stimulated cells (lane a). Unstimulated extracts show an identical pattern, with no change in relative intensity, as do extracts from HeLa and K-562 cells (data not shown). The top two bands in lane a are specific, as they compete with excess unlabeled probe (lane b) but do not compete with unrelated DNA (lane c). Methylation interference analysis performed on the lower of the top two bands shows binding over the first nucleotides of the CRE/ATF-like site and the additional upstream nucleotides that are homologous to OCT-1 motif in the IL-2 promoter (Fig. 4). Interestingly, we consistently see increased cleavage at an adenine residue (highlighted with an asterisk) within this OCT-1 homology domain. Finally, the least retarded band in lane a appears nonspecific, as it does not compete very efficiently (lanes b and c) and does not yield a reproducible pattern of methylation interference (data not shown). Interestingly, none of these bands compete with excess unlabeled CK-1 and CK-2 oligonucleotides (data not shown).

The gel-retardation pattern obtained with a probe extending from −173 to −315 reveals three bands (Fig. 5): a middle band that shows no change in intensity with stimulated (lane S) or nonstimulated extracts (lane U) of MLA 144 cells, in contrast to the top and bottom bands, which do. Methylation interference experiments reveal that the top band represents binding over the AP-1 site. The more intense band, in the middle, involves a particular DNA site downstream of AP-1, which we have called NIP. It is the only DNA-binding seen over the inhibitory region. The bottom band also involves the AP-1 site and may represent a proteolytic product of the top band (data not shown). DNase 1 footprinting experiments (Fig. 6) performed with the same probe confirm that the AP-1-binding site is protected and that this protection is enhanced with extracts from stimulated MLA 144 cells (lanes c, d, and e). In addition, we see a more subtle footprint over the NIP site.

DISCUSSION

Molecular cloning of a variety of hematopoietic growth factors has clarified their biological action, making possible the study of the effects of purified recombinant factors on progenitor cells *in vitro* (4). To apply the lessons learned from *in vitro* studies to an *in vivo* system, it is imperative to learn

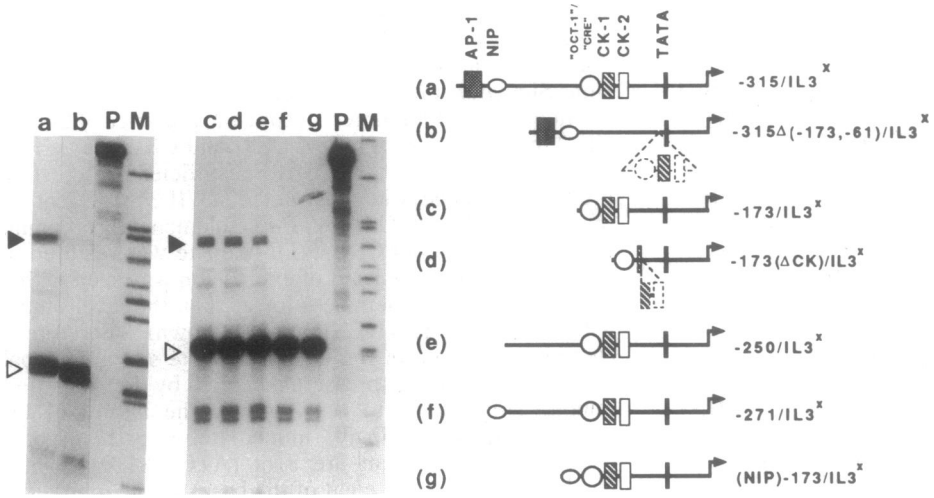

FIG. 3. Role of AP-1, CK-1, CK-2, and NIP in IL-3 expression. Constructs (lanes a–g) are diagramed next to their respective lanes. Deletions are indicated by stippled lines. Lanes a–b and c–g represent two independent experiments. Transfections and RNase protection are as described.

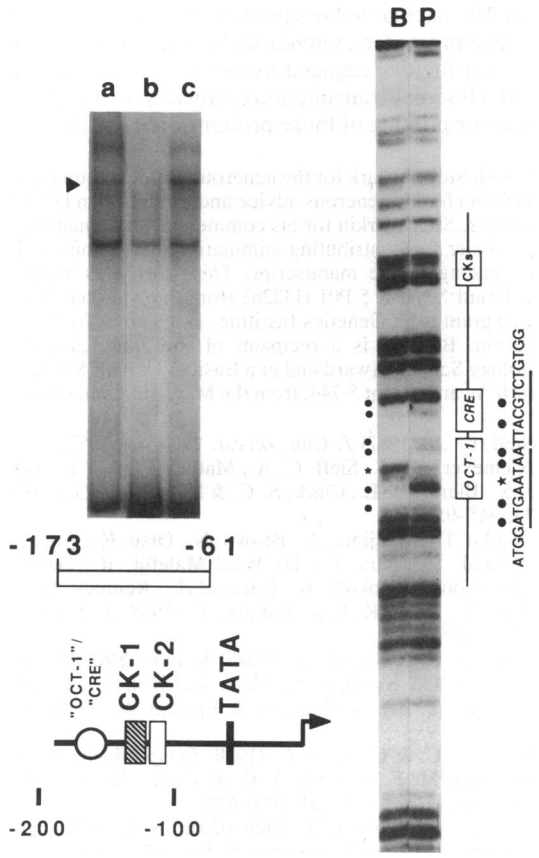

FIG. 4. DNA bindings to activation region 1 of the IL-3 promoter. Each 15-μl reaction contained 1 μl (≈5 μg) of crude nuclear extract from PMA-stimulated MLA 144 cells and 2.5 μg of poly(dI·dC) in buffer B (10 mM Hepes, pH 7.8/50 mM potassium II glutamate/5 mM $MgCl_2$/1 mM EDTA/1 mM dithiothreitol/5% glycerol) with or without self or nonself competitor DNA. Lanes: a, no DNA competitor; b, 100 ng of (−61, −173) DNA; c, 100 ng of (−173, −283) DNA. Retarded band indicated with closed arrowhead was subjected to methylation interference. Positions for the regions of homology with the CRE/ATF and with OCT-1 motifs are shown, and their nucleotide sequences are underlined. B, bound probe; P, free probe. Contacted nucleotides (decreased cleavage) (●) and increased cleavage (★) are indicated next to the DNA ladder and on the corresponding sequence.

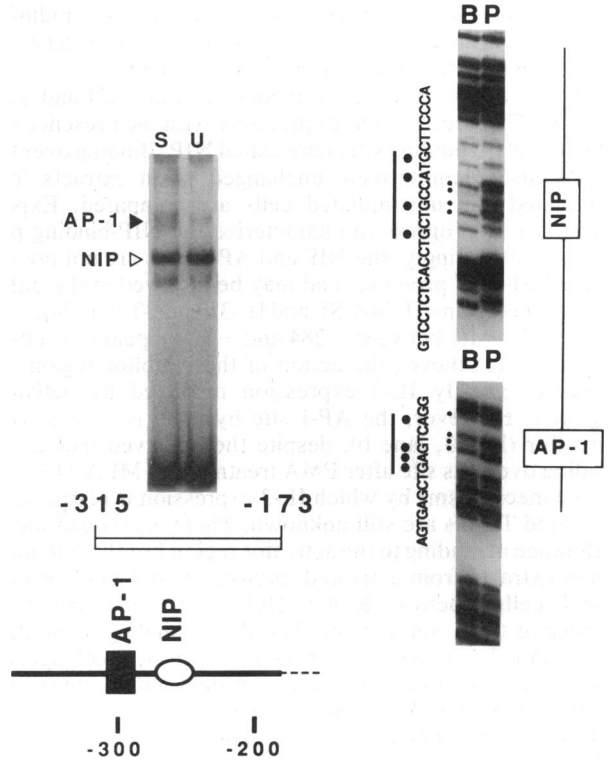

FIG. 5. DNA bindings to probe (−173, −315) in the IL-3 promoter. Binding to the end-labeled probe was done as described in Fig. 4 legend. Lanes: S, extracts from stimulated MLA 144 cells; U, extracts from unstimulated MLA 144 cells. AP-1 and NIP bindings are indicated. Methylation interference for AP-1 and NIP is shown at right. Only the relevant portion of the gel is shown. Positions and sequences for AP-1 and NIP are indicated. B, bound probe; P, free probe. ●, Contacted residues.

more about hematopoietic growth factor gene regulation. To begin such an effort, we have focused on IL-3 because of its involvement in the proliferation and differentiation of early progenitor cells (1).

Human IL-3 is produced by activated T and NK cells, and the expression is regulated, in part, at the transcriptional level (ref. 2 and data not shown). By using a functional assay in which IL-3 reporter genes were transfected into a gibbon T-cell line, MLA 144, we have mapped the transcriptional regulatory elements to a 315-nt sequence upstream of the IL-3 start site. The type of regulation of IL-3 expression unraveled in MLA 144 cells remains to be confirmed in other T cells. However, preliminary experiments in the human T-cell line, Jurkat, are thus far consistent with our results (data not shown).

Activator region 1, located between nt −61 and −173, is essential for IL-3 expression. Deletion of the strongly conserved CK-1 and CK-2 elements present in this region shows that they are neither sufficient nor necessary for IL-3 expression in MLA 144 cells (Fig. 3, lane d). These elements may, however, play an important role in regulation of IL-3 and GM-CSF expression in non-T cells. In contrast, preliminary experiments suggest that a motif (TTACGTCT) closely related to the established CRE/ATF site (15) together with nucleotides immediately upstream are crucial for IL-3 expression. The homology noted between these immediately upstream nucleotides and the OCT-1 motif in the IL-2 promoter is intriguing. Further support for a possible regulatory role of this region comes from DNA-binding experiments, which localize specific contact of a nuclear protein complex over the CRE/ATF-like site and the putative OCT-1 site. However, this binding appears quantitatively unaffected by

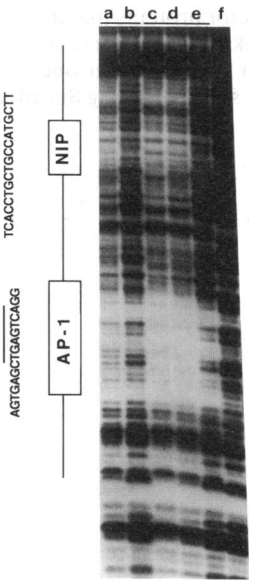

FIG. 6. DNase 1 protection over AP-1 and NIP. The 50-μl reactions contained 0.5 ng (2.5×10^4 dpm) of end-labeled (−173, −315) DNA, 2.5 μg of poly(dI·dC), 50 μg of crude nuclear extract in buffer B containing 1 mM EDTA and 2% (vol/vol) polyvinyl alcohol. After phenol/chloroform extraction, samples were resuspended in formamide/dyes solution and electrophoresed through 8% Tris borate EDTA/urea sequencing gels. Lanes: a and b, extracts from unstimulated cells; c, d, and e, extracts from stimulated MLA 144 cells; f, no nuclear extract. DNase 1 (Boehringer Mannheim) concentrations were as follows: 20 ng/ml (lane c), 70 ng/ml (lanes a, d, and f), and 200 ng/ml (lanes b and e).

the state of T-cell activation. Therefore, if this binding is crucial for transcriptional activation of the IL-3 gene, we must conclude that it is not in itself sufficient.

Strong repressor activity is mediated by nt −271 and −250 (Fig. 3). This area correlates precisely with the presence of a specific DNA-binding site, here called NIP. Binding over this site is also quantitatively unchanged when extracts from stimulated and unstimulated cells are compared. Experiments are in progress to characterize the NIP-binding protein(s). Interestingly, the NIP and AP-1 sites are not present in the GM-CSF promoter and may be involved in the differential expression of GM-CSF and IL-3 in non-T lymphocytes.

The AP-1 site between −284 and −315 appears to serve a dual role. It relieves the action of the inhibitor region and enhances greatly IL-3 expression mediated by activator region 1. However, the AP-1 site by itself is a very weak activator (Fig. 3, lane b), despite the observed increase in binding over this site after PMA treatment of MLA 144 cells.

The mechanisms by which IL-3 expression is restricted to activated T cells are still unknown. There is no quantitative difference in binding to the activator region 1 or the NIP motif when extracts from activated, nonactivated T cells or even non-T cells (such as K-562, HeLa, or COS-7 cells) are compared (data not shown). Therefore, binding alone does not ensure T-cell-specific expression of IL-3; T-cell-specific protein modification or protein–protein interactions, not measured by the DNA-binding assay, must be involved. Although not sufficient for T-cell expression, the increase in AP-1-binding appears to modulate the overall level of IL-3 expression during T-cell activation. Also, the proteins that bind to the three functional regions in the IL-3 promoter must be able to signal to each other. How this is accomplished is unclear. One hypothesis is that the AP-1 complex (18) interacts with the nearby repressor in such a way that binding of this site no longer prevents IL-3 expression. Such a "quenching" model (19) has been described in the repression of yeast a-specific gene expression by the mating-type locus. As already mentioned, the AP-1 complex also signals with activating proteins of region 1 to allow maximal expression of the IL-3 gene. Lastly, the need of the repressor protein may be to ensure that IL-3 expression can only occur when both the AP-1 complex and proteins of activator region 1 are activated.

Some of the functional sites identified are common to other cytokine promoters (e.g., AP-1, OCT-1 in IL-2 promoter) and others are unique (e.g., NIP). The collaboration of common and unique regulatory sites in the promoter of T-cell-derived cytokines has interesting implications. Cell-surface stimulation of T cells may be relayed to effector proteins that interact with common elements of the cytokine promoters. If cytokine expression requires activation of more than one site, added specificity can be achieved by superimposing the effect mediated by nuclear factors interacting with distinct elements in cytokine promoters. Obviously, it will be crucial to identify the nuclear factors engaged in this regulation and to determine which signal-transduction pathways are involved in the synthesis or priming of these proteins after T-cell activation.

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