Multiple Proteins Interact with the Nuclear Inhibitory Protein Repressor Element in the Human Interleukin-3 Promoter*

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T cell expression of interleukin 3 (IL-3) is directed by positive and negative cis-acting DNA elements clustered within 300 base pairs of the transcriptional start site. A strong repressor element, termed nuclear inhibitory protein (NIP), was previously mapped to a segment of the IL-3 promoter between nucleotides −271 and −250. Functional characterization of this element demonstrates that it can mediate repression when linked in cis to a heterologous promoter. DNA binding experiments were carried out to characterize the repressor activity. Using varying conditions, three distinct complexes were shown to interact specifically with the NIP region, although only one correlates with repressor activity. Complex 1 results from binding of a ubiquitous polypeptide that recognizes the 3′ portion of this sequence and is not required for repression. Complex 2 corresponds to binding of transcription factor (upstream stimulatory factor) to an E-box motif in the 5′ portion of the NIP region. DNA binding specificity of complex 3 overlaps with that of upstream stimulatory factor but is clearly distinct. To determine which of the latter two complexes represents NIP activity, we incorporated small alterations into the NIP site of an IL-3 promoter-linked reporter construct and examined their effects on NIP-mediated repression. Functional specificity for repression matches the DNA binding specificity of complex 3; both repressor activity and complex 3 binding require the consensus sequence CTCACNNTNC.

Human interleukin 3 (IL-3) is a potent growth factor, which supports early hematopoietic progenitor cells and potentiates lineage-specific effects of later acting growth factors (1, 2). IL-3 expression is restricted to activated T cells and NK cells (3-6) and is regulated primarily at the level of transcription (7-9). cis-acting promoter elements governing tissue-specific and activation-dependent expression are found within 300 base pairs (bp) of the transcriptional start site (8-13). Promoter deletion experiments have identified two activating sites referred to as ACT-1 (NFIL-3) and ACT-2 (Elf-1) (8, 10, 12). In addition, there is a powerful repressor site, which binds an activity termed nuclear inhibitory protein (NIP) (9). Functional experiments in transfected T cells localized the NIP element between nucleotides −271 and −250 upstream of the transcriptional start (9). These experiments showed that, in the absence of AP-1 and Elf-1 sites, the NIP element blocks activation mediated by the downstream ACT-1 and CBF sites, thus silencing IL-3 expression in activated T cells (9).

The nature of the NIP repressor has remained elusive subsequent to this initial characterization. Studies with the human interleukin 2 (IL-2) promoter identified a repressor site, NRE-A, which interacts with a zinc finger DNA binding protein (14). Since the promoters of the IL-2 and IL-3 genes have common characteristics and function exclusively in activated T cells (5), it was possible that NIP and NRE-A sites might be related. A second possible identity for NIP was suggested by sequence analysis, which revealed that the NIP site contains a consensus E-box sequence (15, 16) that might be recognized by a helix-loop-helix transcription factor.

To better define how NIP interacts with the IL-3 promoter and to characterize the protein mediating repressor activity, we have determined the nucleotide requirements for NIP function. In parallel we have investigated three proteins that bind to the repressor site to determine which correlates with the repressor activity of NIP. Taken together, our results show that NIP and NRE-A are distinct elements and that the repressor protein NIP does not belong to the E-box binding class of transcriptional regulators.

MATERIALS AND METHODS

Cell Lines—The gibbon T cell line MLA 144 (17) was maintained in 5% CO₂ at 37 °C in RPMI medium supplemented with 10% fetal calf serum and antibiotics (50 units/ml penicillin; 50 μg/ml streptomycin). The human J urkat T cell line, the Epstein-Barr virus-transformed Raji cell line, the K562 erythroleukemia cell line, and a murine Abelson-murine leukemia virus-transformed pre-B cell line were cultured in RPMI medium supplemented with 10% fetal calf serum and antibiotics. Mouse erythroleukemia cells were cultured in Dulbecco's modified Eagle's medium supplemented with 2.5% fetal calf serum, 7.5% calf serum, and antibiotics.

Oligonucleotides and IL-3 Promoter Constructs—Sequences of one strand of the double-stranded oligonucleotides used in electrophoretic mobility shift assays (EMSAs) are indicated in Fig. 2. All IL-3 promoter segments are cloned upstream of a reporter gene construct consisting of a modified human IL-3 gene (IL3x) (9). The reporter gene −173/IL3x has been described (9). For (NFIL)-173/IL3x, a double-stranded oligonucleotide spanning nt −267 to −244 of the IL-3 promoter (18) was ligated upstream of −173/IL3x. For −267/IL3x, polymerase chain reaction (PCR) was used to amplify a segment of the IL-3 promoter between nt −267 and −1. The S′ primer used in the reaction incorporated a HindII restriction site for cloning purposes. The PCR fragment was digested with HindII and SmaI (position −61 in the IL-3 promoter) and sub-
cloned into the unique HindIII and Smal sites of the IL-3 promoter fragment in \(-173/1L3^\star\). This resulted in an IL3* reporter gene linked to an IL-3 promoter extending to nt \(-267\). Upstream primers that incorporated mutations in the NIP site listed in Fig. 2 were used on the \(-267/1L3^\star\) DNA template to generate (by PCR) M1, M2, M5, M6, M7, M8, M9, and M10 IL3* reporter gene mutants. Sequence integrity for all new constructs was confirmed by DNA sequencing of the promoter region between the HindIII and Smal sites. All oligonucleotides for PCR and EMSA were obtained from the Oligonucleotide Core Facility at the Dana-Farber Cancer Institute.

Granulocyte-Macrophage Colony-stimulating Factor (GM-CSF) Promoter Constructs—For \(-215^{\star\star}\)IL3*, a fragment of the human GM-CSF promoter from nt \(-215\) to \(-17\) was subcloned immediately upstream of the presumed start site in the IL3* reporter gene at a BanII site. For (NIP)-\(-215^{\star\star}\)IL3*, the same double-stranded NIP oligonucleotide present in (NIP)-\(-173/1L3^\star\) was subcloned upstream of the \(-215^{\star\star}\)IL3* reporter gene. The area of interest was sequenced to confirm the integrity of the construct.

DNA Electroporation—\(4 \times 10^6\) MLA 144 cells were resuspended in 3.5 ml of RPMI medium lacking fetal calf serum. For each electroporation, 50 \(\mu\)g of plasmid DNA was added to the cell suspension and incubated on ice for 5 min. The suspension was split into four aliquots and transferred to disposable cuvettes for electroporation in ProGenex II (Hoefer Scientific Instruments) set at 275 V, 960 \(\mu\)F. Cells were then resuspended in a total of 50 ml of RPMI medium complete with fetal calf serum. After 36–40 h, cells were stimulated with phorbol 12-myristate 13-acetate (10 ng/ml final concentration). Cells were harvested, and RNA was isolated as described (5).

RNA Isolation and RNase Protection Assay—Total RNA was isolated, and expression of the reporter genes was analyzed by an RNase protection assay (9). The probe used was complementary to the first exon of the IL-3 reporter gene, including an insertion of a 12-bp XbaI linker at a HindIII site. After ribonuclease A and T1 digestion, transcripts corresponding to the IL-3 reporter genes protect a 226-nt fragment, whereas endogenous MLA 144 IL-3 transcripts (lacking the 12-bp linker) give rise to two protected fragments of 151 and 63 nt, respectively (9).

EMSAs—Crude nuclear extracts were prepared from different cell lines by the Dignam procedure (19) or a modified procedure by Andrews and Faller (20). T cell extracts (unstimulated and stimulated) were prepared from the Jurkat and MLA 144 cell lines. Cells were stimulated with 10 ng/ml phorbol 12-myristate 13-acetate and 0.5 \(\mu\)g/ml ionomycin for 6 h prior to nuclear extract preparation. Protease inhibitors (from Boehringer Mannheim and Sigma) were included in all extract preparation steps: 0.2 mM phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml pepstatin A, 0.1 \(\mu\)g/ml leupeptin, and 0.4 \(\mu\)g/ml aprotinin. Nuclear extracts from mouse myotube cells (C2DM) were the kind gift of Dr. S. Skapek, Harvard Medical School. Purified upstream stimulatory factor (USF) from HeLa cells was generously provided by Dr. D. Fisher (Dana-Farber Cancer Institute). Oligonucleotide duplexes were labeled with \(\alpha\)\(\mbox{\textbf{P}}\)\(\mbox{-}\)\(\text{TCTP}\) by filling in sticky ends with Klenow polymerase. Labeled oligonucleotides were purified by electrophoresis through nondenaturing polyacrylamide gels. Bands were excised, and oligonucleotides were eluted, precipitated, and resuspended in H\(_2\)O. Oligonucleotide probes were incubated with nuclear proteins at a concentration of approximately 100 fmol/15 \(\mu\)l assay. Two different buffers were used for the binding reaction: buffer A (10 mM Hepes/NaOH, pH 7.8, 50 mM potassium glutamate, 5 mM MgCl\(_2\), 1 mM dithiothreitol, 5% (v/v) glycerol, 1 mM EDTA, and 1 \(\mu\)g/15 \(\mu\)l poly(dI-dC)) and buffer B (10 mM Tris/HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% (v/v) glycerol, 5 mM dithiothreitol, 20 \(\mu\)M ZnSO\(_4\), and 1 \(\mu\)g/15 \(\mu\)l poly(dA)poly(dT)). Nuclear extracts were incubated at a concentration of about 5 \(\mu\)g of total protein assay with radiolabeled oligonucleotide probes for 20 min at 22°C. Competition experiments were performed by adding excess unlabeled oligonucleotides to the binding reaction (at 40 ng/15 \(\mu\)l, a 50–100-fold excess), 15 min prior to adding the probe. The unrelated oligonucleotide (non-specific) used in competition was AGCTTAGCCTCTGTTGATC. Samples were electrophoresed at 22°C through 5% nondenaturing polyacrylamide gels in 0.5 x TBE. Gels were dried on Whatman 3MM paper and used to expose Kodak x-ray film with an intensifying screen.

Immunodot Assays of Basic Helix-Loop-Helix Proteins—\(\alpha\)\(\mbox{\textbf{E}}\)\(\mbox{-}\)\(\alpha\)\(\mbox{\textbf{2}}\) antisera was generously provided by Dr. C. Murre (UCSD). Monoclonal antibody preparations recognizing E2-2, E2-2/E12, and E12/E47 were provided by Pharmingen Inc. (San Diego, CA). Polyclonal rabbit IgG recognizing a 20-amino acid C-terminal peptide of USF and USF from Santa Cruz Biotechnology, CA. Anti-USF antibody (0.05 \(\mu\)g) was incubated at 22°C in 15 \(\mu\)l of binding buffer plus nuclear extract for 1 h prior to addition of the radiolabeled oligonucleotide probe. Antibody specificity was confirmed by preincubation with 1 \(\mu\)l of the 20-amino acid C-terminal USF peptide for 5 h at 22°C before proceeding with the binding reaction as described above.

RESULTS

The NIP Element Is a General Repressor Site—Deletion analysis of the IL-3 promoter revealed the existence of a silencing element, NIP, between bp \(-267\) and \(-244\) (8, 9). To determine whether this region exhibits a repressor function outside of its usual context, it was placed upstream of a truncated version of the IL-3 and GM-CSF promoters (Fig. 1, A and B). The activity of these constructs was measured by RNase protection after transient transfection into the gibbon T cell line MLA 144, as described previously (9). Expression directed by the truncated \(-173\) IL-3 promoter (\(-173/1L3^\star\) reporter gene) was completely silenced when the NIP element was placed just upstream of it (Fig. 1A, lanes a and b). Consistent with the IL-3 promoter results, expression of the reporter gene was drasti-
Characterization of the NIP Repressor Site

To analyze formation of complexes 1 and 2 over the NIP region, we carried out EMSAs under a variety of conditions. There was a striking difference in the pattern of complexes detected when poly(dA)-poly(dT) was used as nonspecific carrier DNA in place of poly(dI-dC). As shown in Fig. 3D, an additional complex (complex 3) was detected using the 5' portion of the NIP sequence, which migrated differently from complex 2 described above. The assignment of the slower migrating band as complex 2 is based on competition experiments using a variety of mutated probes (Figs. 2 and 8 and data not shown). The faster migrating band in Fig. 3D is not consistently seen in our different nuclear extracts (see Fig. 8), and it cannot be fully competed by excess unlabeled oligonucleotide. Thus, three distinct, specific complexes (complexes 1, 2, and 3) are reproducibly detected over the NIP site.

Binding of Complex 1 Is Not Necessary for Repression—The most prominent complex detected with the NIP probe is complex 1. To examine further the DNA binding specificity and function of complex 1, a mutant oligonucleotide was prepared in which four bp were deleted from the 3' portion of the NIP sequence (mutant NIP-Δ; Fig. 2). As shown in Fig. 4A, this oligonucleotide did not compete for complex 1 binding when added in excess and failed to bind complex 1 when radiolabeled and used as a probe. Binding of complex 2 to NIP-Δ was unaffected, but detection required a much longer exposure than that shown for this experiment (data not shown).

The NIP-Δ mutation was incorporated into the −267/IL3 probe to determine whether loss of complex 1 binding correlated with a change in repressor activity at the NIP site. Its activity was compared with −267/IL3 (wild-type), which shows repression of the reporter gene, and a construct in which the NIP site has been deleted, −250/IL3 (NIP minus), which shows no repression. As displayed in Fig. 4B, the 4-bp NIP-Δ deletion had no effect on repression function. Thus, complex 1 does not correlate with NIP activity, and its binding is not required for repression to take place.

Complex 2 is an E-box Complex Immunologically Related to USF—To characterize the DNA binding specificity exhibited by complex 2, several mutated oligonucleotides (listed in Fig. 2) were used to compete binding of complex 2 to the NIP-5' probe (Fig. 5). This experiment demonstrates that the integrity of the E-box consensus sequence in the NIP-5' probe is essential for complex 2 binding. Indeed, a −267/IL3 reporter construct was used to determine whether loss of complex 1 binding correlated with a change in repressor activity at the NIP site. Its activity was compared with −267/IL3 (wild-type), which shows repression of the reporter gene, and a construct in which the NIP site has been deleted, −250/IL3 (NIP minus), which shows no repression. As displayed in Fig. 4B, the 4-bp NIP-Δ deletion had no effect on repression function. Thus, complex 1 does not correlate with NIP activity, and its binding is not required for repression to take place.

To analyze formation of complexes 1 and 2 over the NIP region, NIP-3' and NIP-5' probes (Fig. 2) were used in EMSA (Fig. 3, B, C, and D). The NIP-3' probe specifically bound complex 1 (Fig. 3B), while the NIP-5' probe failed to do so (Fig. 3C). Instead, NIP-5' bound complex 2. Complex 2 is specific and often gives rise to two bands, one major band and a slightly faster migrating minor band. The two bands exhibit identical DNA binding specificity in EMSA competition experiments (Fig. 5 and data not shown).

To maximize detection of all proteins binding to the NIP region, we carried out EMSAs under a variety of different conditions. There was a striking difference in the pattern of complexes detected when poly(dA)-poly(dT) was used as nonspecific carrier DNA in place of poly(dI-dC). As shown in Fig. 3D, an additional complex (complex 3) was detected using the 5' portion of the NIP sequence, which migrated differently from complex 2 described above. The assignment of the slower migrating band as complex 2 is based on competition experiments using a variety of mutated probes (Figs. 2 and 8 and data not shown). The faster migrating band in Fig. 3D is not consistently seen in our different nuclear extracts (see Fig. 8), and it cannot be fully competed by excess unlabeled oligonucleotide. Thus, three distinct, specific complexes (complexes 1, 2, and 3) are reproducibly detected over the NIP site.

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interaction appears to be specific, since preincubation of the antiserum with excess peptide against which it was raised largely restores binding of complex 2 with the NIP-5 probe (Fig. 6A). The E-box in the NIP-5' probe differs from the previously described USF consensus sequence (22). We therefore used a probe containing a bona fide USF binding site (from the adenovirus major late promoter) to confirm our initial observation. Using the USF probe under identical conditions, a complex with the same mobility as complex 2 (Fig. 6, A and B) can be detected. However, the affinity of the binding to the USF
Complex 2 binding consensus: NIP-C3

**Fig. 5.** Binding specificity of complex 2 is identical to the E-box consensus. Nuclear extract from unstimulated MLA 144 cells was used in an EMSA with the NIP-5’ probe. The binding reaction was performed in buffer A in the absence or presence of various competitors, as indicated above each lane. These conditions preclude detection of complex 3 (see Fig. 3, C and D). Sequences of the competitors used are given in Fig. 2. Identical results were obtained with nuclear extracts derived from Jurkat cells. The deduced binding consensus for complex 2 is shown at the bottom.

The NIP-5’ oligonucleotide duplex and four informative mutant probes were used in EMSA to identify a protein complex that showed a binding pattern matching the NIP functional consensus. The NIP-5’ wild-type probe as well as mutant M6 and M8 probes should bind the repressor complex, while the mutant M1 and M9 probes should not. In addition, probe M8 can help discriminate between E-box binding proteins (which cannot be the repressor) and the NIP repressor complex itself. Only complex 3 fulfills the above requirements (Fig. 8A). Taken together, these results indicate that complex 3 is the NIP repressor complex.

Complex 3 Is the NIP Repressor—Having excluded complex 2/USF as the repressor, we turned our attention to complex 3. The NIP-5’ oligonucleotide duplex and four informative mutant probes were used in EMSA to identify a protein complex that showed a binding pattern matching the NIP functional consensus. The NIP-5’ wild-type probe as well as mutant M6 and M8 probes should bind the repressor complex, while the mutant M1 and M9 probes should not. In addition, probe M8 can help discriminate between E-box binding proteins (which cannot be the repressor) and the NIP repressor complex itself. Only complex 3 fulfills the above requirements (Fig. 8A). Taken together, these results indicate that complex 3 is the NIP repressor complex. We have designated this activity NIP-C3, for NIP-complex 3.

The IL-2 promoter, which also directs T cell-specific expression, contains a repressor site, NRE-A, which binds a zinc finger protein (14). Comparison of the complex(es) binding to the NRE-A and NIP sites showed them to have different mobilities in EMSA. Furthermore, NIP oligonucleotides did not compete specific binding to the NRE-A probe; nor did NRE-A oligonucleotides compete NIP-C3 binding to its cognate site (data not shown). This suggests that the IL-2 repressor and NIP-C3 are distinct from each other.

**NIP-C3 Can Be Detected in Different Cell Types and Organisms**—To further characterize NIP-C3, nuclear extracts from various primate and murine cell lines were tested with the same set of probes (Fig. 8, A and B). NIP-C3 was detected in all cases. An equivalent amount of this complex is present in nuclear extracts from unstimulated and stimulated human Jurkat T cells (Fig. 8, A and B). As already shown, NIP-C3 is detected in nuclear extracts from the gibbon T cell line MLA 144. The prominent complex that migrates ahead of NIP-C3 is unlikely to be a breakdown product of NIP-C3 since it binds to probe M9. Its significance, if any, is unknown. NIP-C3 is also detected in erythroleukemic K562 cells, mouse erythroleukemia cells, human B cells (Raji), mouse pre-B cells (2M3), and mouse myeloma (2D8) (Fig. 8B). Although the relative amount of NIP-C3 varies from cell line to cell line, NIP-C3 migration appears to be the same in all preparations, with the exception of the faster component in the NIP-C3 doublet in mouse pre-B cells. This component may have arisen from proteolytic degradation or may represent an altered form of NIP-C3.

**Discussion**

The presence of a negative regulatory element within the IL-3 promoter was originally recognized when a truncated IL-3 promoter lacking the AP-1/ElF-1 sites was tested in T cell lines (8, 9, 12). These experiments localized this element between nt −244 and −270 of the IL-3 promoter and showed that it also functions as a repressor in primary T lymphocytes (23), B cells, and HeLa cells (12). We have shown that the repression function of the NIP region (−267 to −244) is preserved when placed in a different location within the IL-3 promoter or transferred in cis to a heterologous promoter (Fig. 1). Thus, repression through the NIP site is not cell-restricted and may play a more general role than the one involved in IL-3 regulation.
The NIP region was used as a probe to identify proteins that might mediate its repression function. Using various assay conditions for DNA binding in vitro, we were able to detect three specific complexes (Fig. 3). The most prominent complex is complex 1. Partial characterization and purification of complex 1 indicate that it is a ubiquitous protein of with a molecular mass of 63 kDa. It forms on the 3' half of the NIP site and is dispensable for repression function (Fig. 4). Its role in IL-3 regulation remains unknown. A complex with similar properties has been described, which binds a site in the stromelysin gene promoter and appears to mediate phorbol ester activation in cooperation with a nearby AP-1 site (24).

The 5' half of the NIP site interacts with two distinct protein complexes to form complex 2 and complex 3. Both complexes are expressed ubiquitously (Fig. 8 and data not shown) and appear to be conserved across different species. Since a consensus sequence for an E-box binding protein is present in the 5' half of the NIP site, we sought to determine whether either of the two complexes might be a member of the basic helix-loop-helix family of transcription factors. Eleven mutant oligonucleotides of the NIP-5' probe were tested as competitors in EMSAs revealing that the binding specificity of complex 2 (CACNTG) matched the general consensus sequence of an E-box site (CANNTG). The identity of complex 2 was explored using antibodies directed against ubiquitous E-box proteins. Complex 2 was disrupted by an antiserum recognizing the general transcriptional activator USF. Additional experiments established that complex 2 is identical or closely related to USF.

Complex 2 cannot be the NIP repressor, however, since its DNA-binding specificity differs from the functional repressor consensus sequence CTCACNTNC. Other E-box binding proteins are also unlikely candidates, since a mutation altering the invariant G in the NIP E-box core had no effect on repressor function. Since neither complex 1 nor complex 2 binding correlated with repressor activity, we focused on complex 3, the only remaining candidate for this function. The use of different informative mutant oligonucleotides as DNA probes in EMSAs revealed that the binding specificity of complex 3 was not identical to the NIP repressor. Instead, complex 3, designated NIP-C3, displayed the appropriate specificity for being the repressor.

NIP-C3 is expressed in a wide array of tissues and species (Fig. 8 and data not shown). Its binding in vitro is exquisitely sensitive to the type of nonspecific DNA present in the binding
reaction. We were unable to detect NIP-C3 when poly(dI-dC)

was used as carrier. This behavior is reminiscent of the zinc

finger transcription factor ëEF.1, which was originally identi-

fied as a transcriptional activator of the ë-crystallin enhancer

and was subsequently found to repress E2-box-mediated gene

activation through competition of binding. Although NIP-C3

and ëEF.1 share similar binding specificities, they differ in

recognition of at least one important position, where the

presence of a specific nucleotide inactivates NIP repressor

function while it favors ëEF.1 function (25). Although the two

proteins may share similar mechanisms of repression, the

above difference strongly argues that the two factors are

different.

There is accumulating evidence that a growing number of

proteins function by displacing helix-loop-helix transcription

factors from their cognate E-box sequences. Indeed, another

zinc finger protein, ZEB, was recently shown to be able to

silence the IgH enhancer by displacing the E2A complex from

its cognate site (26). Since binding sites for complex 2/USF and

the repressor significantly overlap, a dynamic equilibrium of

occupancy by complex2/USF and NIP-C3 over the 5' portion

of the NIP site may dictate appropriate IL-3 expression in an

analogous manner (27).

Why should the IL-3 promoter contain a strong repressor

element? IL-3 is a potent growth factor, which plays multiple,

complementary roles in normal hematopoiesis (1). Meticulous

control of its production may be necessary to prevent unbridled

proliferation of progenitor cells and to meet the body's fluctu-

ating needs for differentiated cells. IL-3 may be an example of

a protein that is so deleterious when overproduced that block-

ing its expression may be of greater importance than activating

its expression. It has previously been shown that the proximal

portion of the IL-3 promoter activates a basal level of transcrip-

tion in a variety of cell types (12), yet IL-3 is produced only by

activated T cells (5). The NIP repressor may serve as a clamp

that normally prevents IL-3 transcription in all cells. Its effect,

however, can be specifically abrogated when the upstream

AP-1 and Elf-1 sequences engage proteins expressed only in

activated T cells. Future studies must be aimed at understand-

ing the mechanism by which factors acting at the AP-1 and

Elf-1 sites relieve NIP repression.

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