Regulation of c-myc expression is known to occur at the level of transcription initiation. However, the participating promoter elements and their cognate binding proteins have not been fully characterized. c-myc transcription can be stimulated by a number of cytokines including interleukin-2 (IL-2). We have identified a novel IL-2-responsive element, located in the 5’-flanking region of the c-myc gene, between nucleotides -1406 and -1387 (relative to the P2 promoter). This element belongs to the family of interferon-γ activation site-like responsive elements and has the core sequence TCC-CAATAA. We confirmed that IL-2-mediated signaling involves activation by phosphorylation of Jak2 tyrosine kinase and subsequently STAT4. The transcription factor STAT4 binds the TCCCAATAA motif within this responsive element and, therefore, is probably involved in enhancing c-myc transcription upon IL-2 stimulation. Our results propose participation of Jak2 and STAT4 in IL-2-induced up-regulation of c-myc.

The c-myc proto-oncogene is involved in controlling cell proliferation and differentiation (1). The human c-myc gene is composed of three exons (2). The first exon is untranslated and contains two principal promoters: P2, which is the predominant c-myc promoter, generating up to 90% of all transcripts, and P1, which is separated from P2 by 165 bp and generates 10–25% of c-myc RNA (3). All minor promoters, including P0 (located 750 bp 5’ of P2), P3 (in the first intron), and an antisense promoter (found in the second intron), generate less than 5% of c-myc mRNA. The human c-myc gene encodes two polypeptides with apparent molecular masses of 64 and 67 kDa (4). The c-Myc proteins contain a basic region, which mediates sequence-specific DNA-binding, and also helix-loop-helix and leucine zipper motifs, which promote protein-protein interactions (1). Heterodimers formed by MYC and its partner MAX are able to bind DNA and function as transcription activators (5), while MAX homodimers act as transcriptional repressors (5).

The role of c-myc in the development of neoplasia is now well established (3–4, 6–10). The c-myc locus is interrupted by reciprocal chromosomal translocation in human Burkitt’s lymphoma and murine plasmacytomas (11). c-myc is amplified in some myeloid leukemia cell lines and in some cases of human breast cancer (7). The c-myc gene is capable of inducing multiple neoplasms in transgenic mice when fused to immunoglobulin enhancers (6) or a mouse mammary tumor virus long terminal repeat (9). Collectively, each of these perturbations results in the constitutive activation of c-myc transcription.

Unlike the ras gene family, mutations within the coding sequences appear not to be an important feature in converting myc from a proto-oncogene to an oncogene. Rather, abnormally high transcription of c-myc, at an inappropriate stage of the cell cycle or during differentiation, leads to oncogenic transformation (2). Therefore, to define the precise role of the c-myc gene in tumorigenesis, a better understanding of its regulation is warranted. Regulation of c-myc expression is extremely complicated and may occur at the levels of transcription initiation (12), transcript elongation (3, 13), and messenger RNA stability (10). At the level of initiation of transcription, regulation appears to occur via cis-acting regulatory elements (2,11). Although two enhancer elements have been described, 3’ of the c-myc exons (14, 15), the majority of cis-acting regulatory sequences have been identified within the 5’-flanking domain of the human c-myc gene (11, 16). Two sequences, ME1a1 and ME1a2, are located between the principal c-myc promoters P1 and P2. A regulatory region close to P2 (−58 to −68) was found to mediate activation of the P2 promoter by E2F (17, 18) and by the product of the RBl gene (19). A palindromic purine/pyrimidine-rich positive regulatory element, also described as DNase I hypersensitivity site III, has been identified in positions −142 to −115 relative to the P2 promoter (20, 21). A negative regulatory element located between bp −293 and 253, relative to the P1 promoter, has been shown to interact with a transcription factor complex formed by Fos, Jun, and octamer-binding factors (22, 23). An additional regulatory element about 2.2 kilobase pairs upstream of P1 was found to bind nuclear proteins (24, 25). However, all promoter elements and their cognate binding proteins that are necessary for optimal transcription initiation have yet to be fully characterized. Furthermore, the significance of many of these binding sites and their corresponding factors, during physiological regulation of c-myc expression, remains largely unknown.

c-myc is known to be a cytokine-responsive gene (26). Among several cytokines, interleukin-2 (IL-2) is one of the critical regulators of proliferation and differentiation of hematopoietic cells. The functional interleukin-2 receptor (IL-2R) consists of three subunits: the IL-2Rα, IL-2Rβ, and IL-2Rγ chains. Both IL-2Rβ and IL-2Rγ subunits are required to transmit the IL-2 signal to the cell interior (27, 28). The membrane-proximal cytoplasmic region of IL-2Rβ, termed the serine-rich region, has been shown to play a critical role in IL-2-mediated c-myc induction followed by cell proliferation (27, 28). Recently, the
role of IL-2 in stimulation of c-myc transcription has been confirmed (26, 29–31). IL-2 has been shown to selectively stimulate transcription from the P2 promoter (8, 32). However, the participating IL-2 response elements and their cognate binding proteins have not yet been identified.

Previously, we determined that c-myc transcription is rapidly induced in the Natural Killer cell line NK3.3 in response to exogenous IL-2 (33). Accordingly, NK3.3 cells have been chosen as the model IL-2-responsive cell line in our experiments. Using a functional reporter gene assay, we have found an IL-2-responsive element within 537 bp of the 5′-flanking region of the c-myc locus (from bp −1429 to −892 relative to the P2 promoter). Analysis of protein-DNA interactions within this 537-bp region has localized the IL-2-inducible response element and identified its binding protein.

**EXPERIMENTAL PROCEDURES**

**Reagents, Enzymes, and Cytokines—**Cell culture medium (RPMI) and fetal calf serum were purchased from Life Technologies, Inc. Lymphocult was obtained from Biotest (Dreieich, Germany), and recombinant IL-2 was from Genzyme (Cambridge, MA). Poly(dI-dC) and protein A-Sepharose Fast Flow were purchased from American Radiolabeled Biotech. High pressure liquid chromatography-purified oligonucleotides were obtained from Bio-Synthesis (Lewisville, TX). Antibodies to STAT proteins (supershift quality, concentration of 1 mg/ml) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies to anti-phosphotyrosine for Western blotting were obtained from Upstate Biotechnology, Inc. (Lake Placid, NY). All restriction endonucleases, T4 polynucleotide kinase, calf intestine alkaline phosphatase, and DNase I were purchased from New England Biolabs (Beverly, MA). All reagents for PCR were from Promega (Madison, WI).

**Cell Lines and Cultures—**Natural Killer cell line NK3.3 was used as a model in all experiments. Cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, penicillin, streptomycin, and 1-glutamine. Cells were kept in a humidified incubator at 37°C in 5% CO2. Prior to stimulation with IL-2, NK3.3 cells were starved for 18 h in the same medium with a decreased concentration (5%) of serum and without Lymphocult. For the purposes of the experiment, cells were stimulated for 1 h with recombinant IL-2 (200 units/ml).

**Transient Transfection and Reporter Assays—**A variety of DNA fragments were generated from exons 1 and upstream 5′-flanking sequences of the c-myc locus (Fig. LA) and cloned upstream of the promoter-luc transcriptional unit into luciferase reporter construct pGL3-promoter vector (Promega). NK3.3 cells (7–10 × 103 cells/sample) were transiently transfected with 10 μg of the appropriate recombinant vector by electroporation on 800 microfarads at 300 V using “CellPorator” (Life Technologies). All transfections were normalized to β-galactosidase activity by cotransfection of 0.5 μg of a β-galactosidase (pBluescript SK+) expression vector. Transfected cells were starved for 18 h, as described earlier, and then were split in two sets. One set of cells was left untreated; another was transfected with recombinant IL-2 (200 units/ml). In 48 h, all cells were harvested for luciferase and β-galactosidase assays, which were performed according to the manufacturer’s protocols (Promega and ICN Pharmaceuticals, Inc. [Costa Mesa, CA], respectively). The light intensity was measured with a luminesimeter. To exclude variation due to differences in transfection efficiency, signals obtained with the reporter genes were normalized to the levels of the internal β-galactosidase control at each point. The statistical analysis of the data was performed using “Origin” software (Microcal, Northampton, MA).

**Generation of Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA)—**Generation of the nuclear extract from control and IL-2-stimulated NK3.3 cells was performed according to the procedure described by Marzluff (34) and Peterson (35) with a modification that included extraction of nuclear proteins with 0.4M KCl. The protein concentration was determined by using the Bio-Rad protein assay and bovine serum albumin (Sigma) as a standard. Three types of the double-stranded 32P-end-labeled DNA probes were used in the experiments. A 537-bp probe was generated by digestion of the plasmid pMC41-HE (36), containing a 12-kilobase pair c-myc genomic clone, with the restriction endonucleases ClaI and ThhIII. A 100-bp probe was the product of the 537-bp fragment digestion with HpaI. A 20-bp probe was the double-stranded oligonucleotide 5′-GGATTTCCCAATATGAAAAAGC-3′, corresponding to the nucleotide sequence within the C/EBP–HoxP1 fragment.

A mutated 20-bp probe was designed by substitution TTGTT for TTTCC in the original 20-bp probe. The binding reaction, in EMSA studies, used 10 μg of total protein from nuclear extracts and 1 ng (approximately 20,000–30,000 cpm) of the 32P-labeled DNA probe. Incubation was generally performed as described (37) with some corrections. In brief, the binding reaction was carried out for 30 min at 25°C in binding buffer (10 mM HEPES, pH 7.8, 100 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 10% glycerol) in the presence of 2 μg of nonspecific competitor poly(dI-dC). DNA–protein complexes were separated by electrophoresis on 3.5% polyacrylamide gel at 250 V. Supershift experiments were performed to determine the nature of DNA-binding transcription factors, present in the nuclear proteins, using specific internal primers (STAT1 to 3′ normal rabbit IgG). 1 μg of the corresponding antibodies was added to nuclear protein samples prior to mixing with the probe and kept for 10 min at 4°C. EMSA was then performed, as described.

The “stairway assay” (modification of standard EMSA for localization of protein/DNA-binding sites in large DNA segments with known sequence) was performed as described by Van Wijnen (38). Briefly, two samples of 537-bp DNA fragment Clal–ThhIII containing a single 5′-32P-labeled terminus were prepared. Aliquots (25 ng) of each probe were separately digested to completion with each of a series of restriction enzymes chosen to shorten the probe subsequently by 100 bp per cut. After organic extraction and ethanol precipitation, the equimolar quantities of these various shortened DNA fragments were dissolved in TE buffer and used as probes for the standard EMSA protocol.

**Site-directed Mutagenesis—**The oligonucleotide sequence TTCC was replaced by TTGTT in the 100-bp probe using the site-directed mutagenesis procedure described by Ling and Robinson (39) and elsewhere. Mutagenesis involved two rounds of PCR using recombinant plasmid pGL3 with a 100-bp insert as a wild type template. First PCR was performed using RVprimer3 from pGL3 plasmid (Promega) as the forward flanking primer and the mutagenic internal primer 5′-TTAT-TAAACAGCGGTCTGATCC-3′ (annealing temperature = 58°C). The product of that reaction, the “megaprimed,” was purified by a PCR purification kit (Promega), and then reamplified using the reverse flanking primer GlpRmer2 (Promega), as a primer for the second PCR (annealing temperature = 53°C). The final PCR product contained the desired mutation (described above) in a particular DNA sequence. The mutated 100-bp fragment was excised from the PCR product by SacI and XhoI restriction endonucleases (Promega) and cloned into pGL3-promoter vector. The presence of the desired mutation was verified by sequencing with the ABI Prism Big Dye Terminator Cycle sequencing Ready Reaction Kit (PerkinElmer).

**DNase I Footprinting Analysis—**The binding reaction was performed as described for electrophoretic mobility shift assay, except the reaction contained 40 μg of the nuclear extract and 4 ng of labeled DNA. After incubation, samples were digested in the same binding buffer containing 1 mM CaCl2 with 0.01–0.9 units of DNase I for 2 min. Digested samples were precipitated with ice-cold ethanol in the presence of saturated ammonium acetate and carrier tRNA, washed twice with 70% ethanol, and resuspended in the electrophoresis loading buffer to 10,000 cpm/μl. Equal loads were counted onto 6% acrylamide/8 M urea sequencing gel. The relative intensity of radioactive bands was determined by PhosphorImager analysis, utilizing a PhosphorImager SI (Molecular Dynamics, Inc., Sunnyvale, CA).

**Scanning Densitometry—** Autoradiographed bands were quantified within the linear range of film on a model 300A laser densitometer and ImageQuant software (Molecular Dynamics).

**Immunoprecipitation and Western Blotting—**NK3.3 cells (107 cells/sample) were starved for 18 h prior to IL-2 stimulation and then treated with 200 units/ml of recombinant IL-2 for 1 h. Untreated (control) and IL-2-treated cells were lysed in 20 mM Tris buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 10 mM NaF, 1 mM Na3VO4, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. For Immunoprecipitation, lysis supernatants were incubated with 1 μg of specific polyclonal antibodies (anti-JAK2 or anti-STAT4) for 1 h at 4°C. 40 μl of protein A-Sepharose was then added to each sample for an additional incubation, with agitation, at 4°C for 1 h. Precipitated proteins were separated on 7.5% Laemmli polyacrylamide gels, blotted onto polyvinylidene difluoride membranes, probed with anti-phosphotyrosine antibodies, and followed by horseradish peroxidase-conjugated sheep anti-mouse antibodies. Immunoreactive bands were visualized with chemiluminescent SuperSignal Substrate for Western blotting (Pierce).
results

Identification of a Functional IL-2 Response Element within the 5'-Flanking Sequence of c-myc—To identify the region of the human c-myc gene essential for IL-2-regulated transcriptional activity, various luciferase reporter constructs were assembled. These constructs incorporated the known and putative c-myc regulatory regions, including exon 1 and upstream 5'-flanking sequences (2500 bp linked to the P2 promoter, described in Fig. 1A). These regions were cloned upstream of the SV40 promoter in the luciferase reporter plasmid pGL3-promoter (Promega). Locations of the fragments are marked with arrows. A, a functional IL-2-inducible element has been found within the 537-bp ClaI–ThhIII DNA fragment of the 5'-flanking region of the c-myc gene. NK3.3 cells (7–10 × 10⁶ cells) were transiently cotransfected with 0.5 μg of pRSV-β-galactosidase reference plasmid and 10 μg of the empty pGL3-promoter vector construct (pGL3) or the same constructs containing a 537-bp ClaI–ThhIII DNA fragment in both forward (pGL3/500(+) and reverse (pGL3/500(−)) orientations (described under “Experimental Procedures”). After incubation for 48 h in the absence (light bars) or presence (dark bars) of IL-2, cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. The values shown are averages of four independent experiments ± S.D. and are expressed as relative -fold induction over normalized luciferase values from untreated NK3.3 cells containing the empty vector control only. C, the localization of the IL-2-responsive element within the ClaI–ThhIII DNA fragment of the c-myc gene was confirmed by EMSA with nuclear extracts (NE) from NK3.3 cells. Cells were left untreated (lanes 2 and 4) or were treated with 200 units/ml of recombinant IL-2 for 1 h (lanes 3 and 5). The double-stranded 537-bp probe (ClaI–ThhIII fragment from the plasmid pmc41) was 32P-end-labeled. Lane 1 contains the probe only and no NE. For each binding reaction, 10 μg of total NE protein and 1 ng (approximately 20,000 cpn) of the probe were taken. For the cold competition (lanes 4 and 5), the binding reaction was performed in the presence of 100 ng of the unlabeled probe. The position of the DNA-protein complexes is marked with the arrow.

Precise Localization of the IL-2-responsive Element within the 5'-Flanking Region of c-myc Gene—The EMSA was used to analyze protein-DNA interactions within this putative 537-bp, cis-acting, IL-2-inducible regulatory domain. The end-labeled 537-bp ClaI–ThhIII fragment was incubated with nuclear extracts from untreated and IL-2-stimulated cells and analyzed.

FIG. 1. A, outline of the DNA fragments, generated from exon 1 and upstream 5'-flanking sequences of c-myc locus and inserted upstream of the promoter in the luciferase reporter plasmid pGL3-promoter (Promega). Locations of the fragments are marked with arrows. B, a functional IL-2-inducible element has been found within the 537-bp ClaI–ThhIII DNA fragment of the 5'-flanking region of the c-myc gene. NK3.3 cells (7–10 × 10⁶ cells) were transiently cotransfected with 0.5 μg of pRSV-β-galactosidase reference plasmid and 10 μg of the empty pGL3-promoter vector construct (pGL3) or the same constructs containing a 537-bp ClaI–ThhIII DNA fragment in both forward (pGL3/500(+) and reverse (pGL3/500(−)) orientations (described under “Experimental Procedures”). After incubation for 48 h in the absence (light bars) or presence (dark bars) of IL-2, cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. The values shown are averages of four independent experiments ± S.D. and are expressed as relative -fold induction over normalized luciferase values from untreated NK3.3 cells containing the empty vector control only. C, the localization of the IL-2-responsive element within the ClaI–ThhIII DNA fragment of the c-myc gene was confirmed by EMSA with nuclear extracts (NE) from NK3.3 cells. Cells were left untreated (lanes 2 and 4) or were treated with 200 units/ml of recombinant IL-2 for 1 h (lanes 3 and 5). The double-stranded 537-bp probe (ClaI–ThhIII fragment from the plasmid pmc41) was 32P-end-labeled. Lane 1 contains the probe only and no NE. For each binding reaction, 10 μg of total NE protein and 1 ng (approximately 20,000 cpn) of the probe were taken. For the cold competition (lanes 4 and 5), the binding reaction was performed in the presence of 100 ng of the unlabeled probe. The position of the DNA-protein complexes is marked with the arrow.

FIG. 2. Stairway assay. A, outline of 32P-end labeled probes for EMSA generated by restriction digestion with the selected endonucleases. Numbers of the labeled fragments correspond to the numbers of the lanes in EMSA. B, EMSA for stairway assay with NE from non-stimulated (lanes 2 and 5–10) and IL-2-stimulated (lanes 3, 5a–10a, and 11–16) NK3.3 cells was made for the end-labeled ClaI–ThhIII fragment (lanes 1–3) or for the same fragment labeled only on the ThhIII end (lanes 4–10a) or only on the ClaI end (lanes 11–16). 10 μg of total NE protein and 1 ng (approximately 20,000 cpn) of the probe or the product of its digestion was used for each binding reaction. Lanes 1 and 4 contain the probes only and no NE. Positions of DNA-protein complexes are shown with arrows.
plexes between nuclear proteins and the 32P-labeled portion of the 5′ fragment (lane 6a). Shortening of the probe by 100 bp (lane 6b, cleavage with HpaI) eliminated the band of slowly migrating complex. These data map the position of the protein-binding site within the 100-bp DNA fragment (−1429 to −1329 bp relative to the P2 promoter).

To examine whether this shortened DNA fragment (ClaI–HpaI) contains the same enhancer element(s) responsive to IL-2 activation and is acting in the same manner as the full-length 537-bp probe, a 100-bp fragment was cloned into the pGL3-promoter luciferase reporter construct, and transient transfections were carried out, as described previously. As shown in Fig. 3A, insertion of the ClaI–HpaI fragment in any orientation (forward or reverse) did not activate this reporter in the absence of IL-2. IL-2 induction led to a 12–15-fold increase in luciferase activity using both constructs pGL3/100(+) and pGL3/100(−). These data are statistically significant (p = 0.05). Comparison of luciferase transfection assays performed with reporter constructs containing the 537-bp insert (Fig. 1B) and the 100-bp insert (Fig. 3A) revealed that the smaller insert induced higher levels of transcriptional activation than the larger one. This suggests that replacement of DNA sequence ClaI–TthIII in the reporter gene construct with the smaller ClaI–HpaI fragment eliminated additional negative effects of other regulatory elements in this portion of the c-myc 5′-flanking region.

To confirm the ability of the 100-bp fragment to form the same type of DNA-protein complex as the 537-bp fragment, we performed another EMSA experiment using both fragments as probes. Incubation of each of these probes with nuclear extracts from IL-2-stimulated cells leads to formation of specific DNA-protein complexes (Fig. 3B, lanes 3 and 6). The remainder of
the 537-bp probe (Hpa1–TthIII, 400-bp fragment) was unable to bind nuclear proteins from IL-2-stimulated cells (data not shown).

To locate the position of the protein-binding site within the 100-bp fragment, DNase I footprinting analysis was performed, as described under “Experimental Procedures.” As shown in Fig. 4, only nuclear extracts from IL-2-stimulated cells were able to generate the DNase I-protected region in the 100-bp fragment (lane 7). Using densitometry and PhosphorImager analysis, we found at least 70% reduction of the bands' intensity in the protected region (marked with the arrows) while compared with the bands' intensity outside this area. The arrows indicate the position of the 20-bp protected sequence 5'-GCTTTTCCATAAATAAGG-3' (–1406 to –1387 relative to P2 promoter). Based on this sequence, a 20-bp probe was generated for EMSA experiments. Fig. 5 presents the results of EMSA and competition experiments with 20- and 100-bp fragments. First, the 20-bp probe was able to generate complexes with nuclear extracts from IL-2-stimulated cells (lane 11, marked with the arrows). Minor complexes, other than those indicated by arrows, were not reproducibly induced by IL-2 in multiple experiments and thus should be disregarded. Second, the presence of a 100-fold excess of unlabeled 20-bp fragment removed the complexes formed between nuclear proteins of stimulated cells and the 100-bp fragment (lane 8). Third, the presence of a 100-fold excess of cold 100-bp probe eliminated 82% (determined by densitometry) of the complexes, formed by the 20-bp fragment and nuclear proteins (lane 13). Collectively, these results provide evidence of identical protein-binding sites in both fragments. Identification of the Putative Transcription Factor Associated with (or within) the Characterized 20-bp Protein-Binding Site—Using the data base “Transfac Matrix,” computer analysis of putative binding sites for known transcription factors was performed for the aforementioned 20-bp sequence. A STAT binding-related sequence 5'-TTCCAATAA-3' was found within this area. This region, located between bp –1402 and –1394 relative to the P2 promoter, is highly homologous (approximately 85.6%) to STAT1α and -β, STAT2, STAT3, STAT4, and STAT5 binding sites. Therefore, the ability of anti-STAT antibodies to alter the mobility of slowly migrating DNA-protein complexes in “supershift” experiments was examined. As seen in Fig. 6A, among various anti-STAT antibodies, only the presence of anti-STAT4 antibodies (lane 6) reduced the abundance of the complexes formed between nuclear proteins and 537-bp probe. Identical results were obtained when shortened probes (100 and 20 bp) were

![Image](https://example.com/image.jpg)
could disengage the IL-2-dependent activation of the promoter in vivo, the mutated 100-bp (ClaI-HpaI) fragment was cloned into the luciferase reporter plasmid pGL3-promoter. Using site-directed mutagenesis (40), we mutated the sequence TTCTC to TTGTT in the STAT4 binding site of the 100-bp fragment (for details, see “Experimental Procedures”), introduced this fragment to the pGL3-promoter plasmid, and tested this recombinant construct (pGL3/mut.100) for its ability to respond to IL-2 in the transient transfection assays (as described above). As shown in Fig. 7B, no significant increase over control in luciferase activity was found in either nonstimulated or IL-2-stimulated cells, transfected with the construct pGL3/mut.100. Therefore, we conclude that the construct containing the mutated STAT4 binding site was unable to mediate response to IL-2 in contrast to the pGL3/100 construct containing the wild type consensus element within the 100-bp fragment (see Fig. 3A).

Previous studies have demonstrated that activation of STAT4 is a result of its phosphorylation by activated Jak2 protein tyrosine kinase (41). Accordingly, we examined the ability of IL-2 to induce activation of the Jak2/STAT4 signal transduction pathway. Since tyrosine phosphorylation is critical to the activation of Jaks and STATs, Jak2 and STAT4 were immunoprecipitated with corresponding antibodies from the lysates of IL-2-stimulated NK3.3 cells, and Western blotting with anti-phosphotyrosine antibodies was performed. Phosphorylated Jak2 and STAT4 were found only in IL-2-activated cells (Fig. 8A) and not in untreated cells. These results are consistent with our hypothesis that IL-2 activates the Jak2/STAT4 pathway, and STAT4 binds to an IL-2 response element in the c-myc gene, thereby positively regulating c-myc transcription.

**FIG. 8. IL-2-mediated signaling involves Jak2 and STAT4 activation by tyrosine phosphorylation.** Immunoprecipitation of Jak2 and STAT4 with corresponding antibodies was performed on whole cell extracts from nonstimulated (+) and IL-2-stimulated (-) NK3.3 cells, followed by Western blotting with anti-phosphotyrosine antibodies (A) or with antibodies to Jak2 or STAT4 (B).

**DISCUSSION**

Three distinct signaling pathways, linked to IL-2R, have been recently identified (28, 42): the p56^ck^ pathway leading to c-fos/c-jun induction, the bcl-2 induction pathway, and the c-myc induction pathway. Importantly, none of these pathways affect activation of the other. However, the combination of any two of the three pathways is sufficient to promote cell growth in the absence of cytokines (28). Therefore, it is reasonable to suggest that cross-talk between intermediate members of the different IL-2-mediated pathways could provide sufficient induction of target genes. The induction pathway of the c-myc gene is primarily linked to the serine-rich region of IL-2Rβ chain (27, 28), which is associated with Jak1 (43, 44) and Syk protein-tyrosine kinases (45). However, involvement of IL-2Rγ and association of this subunit with Jak3 in the same transduction pathway has been confirmed (46). Another member of

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**FIG. 7.** A, mutations in the STAT4 consensus binding site eliminate binding between nuclear proteins from IL-2-stimulated cells and the 20-bp probe. EMSA with NE from nonstimulated (lanes 2 and 4) and IL-2-stimulated (lanes 3 and 6) NK3.3 cells was performed for the original (lanes 1–3) and mutated (lanes 3–6) 20-bp probe under conditions, described above, in the legend to Fig. 5. Lanes 1 and 4 have probes only, no NE. The position of the DNA-protein complexes is marked with an arrow. B, mutations in the STAT4 binding site destroy the ability of the pGL3/100bp construct to mediate response to IL-2. The 100-bp ClaI-HpaI fragment was mutated by site-directed mutagenesis (described under “Experimental Procedures”) and cloned into pGL3-promoter vector construct (pGL3/mut.100). The transient transfection experiments and luciferase assays were performed as described above in the legend to Fig. 3A.

To investigate whether the mutations in the STAT element used. Fig. 6B shows that the addition of anti-STAT4 antiserum to 100-bp (lane 4) or 20-bp (lane 9) probes reduced the abundance of slow migrating DNA-protein complexes in a similar manner. Collectively, these results indicate the presence of STAT4 in nuclear extracts from IL-2-stimulated cells and its ability to bind the DNA sequence within the c-myc locus and, therefore, enhance c-myc transcription after IL-2 stimulation.

As described by Yamamoto and co-workers (40) and recommended for use by Santa Cruz Biotechnology, replacement of one or more C for T nucleotides in the STAT4 consensus binding site eliminates binding between this mutated probe and STAT4 protein in the gel shift assay. Thus, we performed another EMSA experiment using two probes: the original 20-bp probe and a mutated one, where part of the consensus binding site TTTCC was changed to TTGTT. Fig. 7A shows that the mutated probe lost the ability to bind STAT4 in IL-2-stimulated cells (lane 6).

To investigate whether the mutations in the STAT element could disengage the IL-2-dependent activation of the promoter in vivo, the mutated 100-bp (ClaI-HpaI) fragment was cloned into the luciferase reporter plasmid pGL3-promoter. Using site-directed mutagenesis (40), we mutated the sequence TTCTC to TTGTT in the STAT4 binding site of the 100-bp fragment (for details, see “Experimental Procedures”), introduced this fragment to the pGL3-promoter plasmid, and tested this recombinant construct (pGL3/mut.100) for its ability to respond to IL-2 in the transient transfection assays (as described above). As shown in Fig. 7B, no significant increase over control in luciferase activity was found in either nonstimulated or IL-2-stimulated cells, transfected with the construct pGL3/mut.100. Therefore, we conclude that the construct containing the mutated STAT4 binding site was unable to mediate response to IL-2 in contrast to the pGL3/100 construct containing the wild type consensus element within the 100-bp fragment (see Fig. 3A).

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**DISCUSSION**

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the family Syk/ZAP70 protein-tyrosine kinase, ZAP70, is also suspected to be IL-2R-associated (42, 47). As yet, no association has been found for Jak2 and IL-2R. The distal components, linking the IL-2 receptor to c-myc transcription, are largely unknown.

Although several positive and negative regulatory elements within the c-myc locus have been described, none have been identified as IL-2-responsive. In addition, no known transcription factor(s) have been shown to be directly involved in IL-2-mediated up-regulation of c-myc. The lack of specific information regarding this important cytokine-mediated effect prompted our search for IL-2-responsive elements within the c-myc locus and the corresponding transcription factor(s).

Computer modeling of the c-myc flanking DNA suggests that two slow-moving DNA fragments, spanning nucleotides (relative to the c-myc promoter P2) −983 to −617 and −1855 to −1219, form large left-handed superhelices or curved structures (48). It is possible that curved DNA segments may play a regulatory role in DNA transcription (48). In the current studies, we first identified a functional IL-2-responsive element within the area −1429 to −892 bp relative to the P2 promoter (partially overlapping with the first regulatory DNA fragment determined by Kumar and Leffak (48)). Then we localized the precise position of this element and determined (using EMSA and DNase I footprinting) its core nucleotide sequence. Finally, STAT4 was identified as at least one transcription factor that binds the core sequence (TTCCAATAA) within this element.

The DNA sequence elements in the promoters of genes that bind STAT proteins can be classified into two groups (49). The prototype of the first class is the interferon-stimulated response element (50). Interferon-stimulated response elements have the consensus sequence AGTTTCNNTTTCN(C/T) where N is any nucleotide. The second class comprises the IFN-γ activation site (GAS)-like response elements. A significant number of GAS-like sequences have been identified in promoters of genes activated by different extracellular signaling proteins. Various STATs have been shown to bind at least one of the GAS-like elements (40, 51–53). All of these elements have the palindromic core sequence TTNNNNAAA, but they differ in five inner nucleotides. Selective and specific activation of genes, by different STAT dimers, with different binding affinities, involves slightly different response elements. We have identified a new IL-2 response element, which also contains the palindromic core sequence TTCCAATAA, belonging to the GAS-like response elements. Under the conditions described, STAT4, upon activation by Jak2, binds to this response element and probably promotes c-myc expression. Until recently, only IL-12 and IFN-α were shown to mediate signaling through the phosphorylation of Jak2/Tyk2 and subsequently STAT4 (54–57). Therefore, overlapping biological responses to IL-2 and IL-12 could possibly be explained by the synergetic effect on IFN-γ production (58, 59) or induction of differential expression of specific sets of genes (54). However, recently K. Wang and co-workers (60) described the direct involvement of IL-2 in the Jak2/STAT4 signaling pathway. They demonstrated the ability of IL-2 to activate target genes through phosphorylation of Jak2 and STAT4 in primary resting T cells or mitogen-activated T cells (60). Thus, K. Wang suggested that this unique activation of the STAT4-signaling pathway only in NK cells might underlie the distinct functional effect of IL-2 on this cell population. Our data generated on the same model of NK cells propose c-myc as one of the target genes in this signaling pathway.

Mechanisms of Jak2/STAT4 involvement in IL-2-mediated c-myc up-regulation are still largely unknown. The activation of STAT4, in response to IL-2, is not due to the autoimmune production of IL-12 or IFN-α, because the presence of IFN-α or IL-12-neutralizing antibodies did not affect the activation of STAT4 in response to IL-2 (60). IL-2R is known to be associated withJak1 and Jak3 kinases but not with Jak2. Although unproved, it is provocative to suggest that some novel docking proteins are required to transmit IL-2-mediated signals to Jak2 kinase. A signal-transducing adapter molecule (STAM), which contains a Src homology 3 domain and immunoreceptor tyrosine-based activation motif (ITAM) could play the role of such a docking protein (61). STAM is associated with Jak2 and Jak3 tyrosine kinases via its ITAM region and phosphorylated by Jak2 and Jak3 upon stimulation with IL-2 and other cytokines. The wild-type STAM, but not STAM mutants deleted of Src homology 3 domain or immunoreceptor tyrosine-based activation motif, significantly enhances c-myc induction mediated by IL-2 (61). Therefore, STAM is considered involved in the IL-2-induced c-myc pathway. These signals are positioned immediately downstream of Jak kinases and potentially could transmit the signals between different Jak enzymes.

Further experiments are needed to identify all intermediates in this signal pathway. However, the data reported in this work represent, to our knowledge, the first demonstration of IL-2 up-regulation of c-myc expression upon activation of the Jak2/STAT4 signaling pathway. We have identified a novel IL-2-responsive element in the 5′-flanking region of c-myc and confirmed the role of STAT4 as the cognate binding protein for this element.

We believe that results of these studies will help in a better understanding of the complex control of c-myc expression and also of various molecular mechanisms by which cytokines control and regulate transcription of proto-oncogenes.

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REFERENCES

1. Henriksson, M., and Luscher, B. (1996) Adv. Cancer Res. 68, 109–182
2. Lang, J. C., Whitelaw, B., Talbot, S., and Wilkinson, N. M. (1988) Br. J. Cancer 58, 62–66
3. Bentley, D. L., and Groudine, M. (1986) Mol. Cell. Biol. 6, 3481–3489
4. Henn, S. R., King, M. W., Bentley, D. L., Anderson, C. W., and Eisenman, R. N. (1988) Cell 52, 185–195
5. Kretzner, L., Blackwood, E. M., and Eisenman, R. N. (1992) Nature 359, 426–429
6. Adams, J. M., Harris, A. W., Pinkert, C. A., Ooreran, L. M., Alexander, W., Leder, A., Saylor, S., Palmeter, R. D., and Brinster, R. L. (1985) Nature 318, 533–538
7. Altalio, K. (1984) Med. Biol. 62, 28–32
8. Hoover, R. G., Kausahl, V., Lary, C., Travis, F., and Snead, T. (1995) Curr. Top. Microbiol. Immunol. 194, 257–264
9. Leder, A., Pattengale, P. K., Ruo, A., Stewart, T. A., and Leder, P. (1986) Cell 45, 485–495
10. Piechaczek, M., Yang, J. Q., Blanchard, J. M., Jeanteur, P., and Marcu, K. B. (1985) Cell 42, 589–597
11. Maruo, K. B., Bossone, S. A., and Patel, A. J. (1992) Annu. Rev. Biochem. 61, 809–860
12. Bentley, D. L., and Groudine, M. (1988) Nature 331, 702–706
13. Bentley, D. L., and Groudine, M. (1988) Cell 53, 245–256
14. Mautner, J., Joos, S., Werner, T., Eick, D., Bornkamm, G. W., and Polack, A. (1995) Nucl. Acids Res. 23, 72–80
15. Murphy, L. C., Huel, N., and Davie, J. R. (1996) DNA Cell Biol. 15, 543–549
16. Miller, T. L., Lin, Y., Sun, J. M., Couts, A. S., Murphy, L. C., and Davie, J. R. (1990) J. Cell. Biochem. 56, 560–571
17. Huesbert, S. W., Lipp, M., and Nevin, J. R. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 3594–3598
18. Thalheimer, K., Sysnovich, H., Mertz, B., Winacker, E. L., and Lipp, M. (1989) Genes Dev. 3, 527–536
19. Hamel, P. A., Gill, R. M., Phillips, A., and Galle, B. L. (1992) Mol. Cell. Biol. 12, 3431–3438
20. Postel, E. H., Mango, S. E., and Flint, S. J. (1989) Mol. Cell. Biol. 9, 5123–5133
21. Siebenlist, U., Hennihausen, L., Battey, J., and Leder, P. (1984) Cell 37, 381–391
22. Hay, N., Takimoto, M., and Bishop, J. M. (1989) Genes Dev. 3, 293–303
23. Takimoto, M., Quinn, J. P., Farina, A. R., Staudt, L. M., and Levens, D. (1989) J. Biol. Chem. 264, 8992–8999
24. Ariga, H., Inamura, Y., and Iguchi-Ariga, S. M. (1989) EMBO J. 8, 4273–4279
25. Iguchi-Ariga, S. M. M., Okazaki, T., Itani, T., Ogata, M., Sato, Y., and Ariga,
Regulation of c-myc Transcription

H. (1988) EMBO J. 7, 3135–3142
28. Reed, J. C, Nowell, P. C., Hoover, R. G. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 4221–4224
29. Hatakeyama, M., Mori, H., Doi, T., and Taniguchi, T. (1989) Cell 59, 837–845
30. Miyazaki, T., Liu, Z. J., Kawahara, A., Minami, Y., Yamada, K., Tsujimoto, Y., Barsoumian, E. L., Permutter, R. M., and Taniguchi, T. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8724–8728
31. Azzoni, L., Anegon, I., Calabretta, B., and Perussia, B. (1995) J. Immunol. 154, 491–499
32. Kerkhoff, E., and Ziff, E. B. (1995) Nucleic Acids Res. 23, 4857–4863
33. Shibuya, H., Yoneyama, M., Ninomiya-Tsuji, J., Matsumoto, K., and Taniguchi, T. (1992) Cell 70, 57–67
34. Broome, H. E., Reed, J. C., Godillot, E. P., and Hoover, R. G. (1987) Mol. Cell. Biol. 7, 2988–2993
35. Bros, H. E., Reed, J. C., Godillot, E. P., and Hoover, R. G. (1987) Mol. Cell. Biol. 7, 2988–2993
36. Kornbluth, J., and Hoover, R. G. (1988) J. Immunol. 141, 3234–3240
37. Marzluff, W. F. (1984) Transcription and Translation, p. 91, IRL Press, Washington, D. C.
38. Marzluff, W. F. (1984) Transcription and Translation, p. 91, IRL Press, Washington, D. C.
39. ar-Rushdi, A., Nishikura, K., Erikson, J., Watt, R., Rovera, G., Groce, C. M. (1993) Science 266, 1045–1047
40. Witthuhn, B. A., Silvennoinen, O., Miura, O., Lai, K. S., Cwik, C., Liu, E. T., and Ihle, J. N. (1994) Nature 370, 153–157
41. Minami, Y., Nakagawa, Y., Kawahara, A., Miyazaki, T., Sada, K., Yamamura, H., and Taniguchi, T. (1995) Immunity 2, 89–100
42. Kawahara, A., Minami, Y., Miyazaki, T., Ihle, J., and Taniguchi, T. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8724–8728
43. Chan, A. C., Desai, D. M., and Weiss, A. (1994) Annu. Rev. Immunol. 12, 555–592
44. Kumar, S., and Lefkak, C. (1989) Nucleic Acids Res. 17, 2819–2833
45. Heim, M. H. (1996) Eur. J. Clin. Invest. 26, 1–12
46. Darnell, J. E., Jr. (1997) Science 277, 1630–1635
47. Hoo, J., Schindler, U., Henzel, W. J., Ho, T. C., Brassuer, M., and McKnight, S. L. (1994) Science 225, 1701–1706
48. Jacobson, N. G., Szabo, S. J., Weber-Nordt, R. M., Zhong, Z., Schreiber, R. D., Darnell, J. E., and Murphy, K. M. (1995) J. Exp. Med. 185, 1755–1762
49. Zhong, Z., Wu, Z., and Darnell, J. E., Jr. (1994) Science 264, 95–98
50. Azzoni, L., Kanakaraj, P., Zabelina, O., and Perussia, B. (1996) J. Exp. Med. 157, 3235–3241
51. Bacon, C. M., McVicar, D. W., Ortaldo, J. R., Rees, R. C., O'Shea, J. J., and Johnston, J. A. (1995) J. Exp. Med. 181, 399–404
52. Bacon, C. M., Pecore, E. F., III, Ortaldo, J. R., Rees, R. C., Larner, A. C., Johnston, J. A., and O'Shea, J. J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7307–7311
53. Trinchieri, G. (1995) Annu. Rev. Immunol. 13, 251–276
54. Trinchieri, G. (1995) Annu. Rev. Immunol. 13, 251–276
55. Aste-Amezaga, M., D'Andrea, A., Kubin, M., and Trinchieri, G. (1994) Cell 156, 480–492
56. Chan, S. H., Perussia, B., Gupta, J. W., Kobayashi, M., Pospisil, M., Yeung, H. A., Wolf, S. F., Young, D., Clark, S. C., and Trinchieri, G. (1991) J. Exp. Med. 173, 869–879
57. Wang, K. S., Ritz, J., and Frank, D. A. (1999) J. Exp. Med. 189, 299–304
58. Takeshita, T., Arita, T., Higuchi, M., Asao, H., Endo, K., Kurosawa, H., Tanaka, N., Murata, K., Ishii, N., and Sugamura, K. (1997) Immunity 6, 449–457