BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **223,** 64–72 (1996)
ARTICLE NO. 0847

An Upstream Positive Regulatory Element in Human GM-CSF Promoter Is Recognized by NF-κB/Rel Family Members

Marco Musso,[1,2] Paola Ghiorzo,[1,3] Paola Fiorentini, Roberto Giuffrida, Paola Ciotti, Cecilia Garré, Roberto Ravazzolo, and Giovanna Bianchi-Scarrà

IBiG, Institute of Biology and Genetics, University of Genova, 16132 Genova, Italy

Received April 24, 1996

To further extend the previous analysis of cis-acting elements and cognate trans-acting factors that contribute to GM-CSF transcriptional regulation, we have examined a promoter region between −1742 and −2010. DNase I footprinting assays showed four protected sequences named A, B, C and D. DNA transfections in the T-lymphoid Mo cell line, which constitutively expresses GM-CSF, indicated that the A element, located between −2002 and −1984, has a positive role on transcription. Further characterization by electrophoretic mobility shift assays in the presence of different competitor oligonucleotides showed that this element binds a factor of the NF-κB/Rel family.

Expression of GM-CSF, a cytokine involved in hematopoiesis and host defense is regulated both at the transcriptional and post-transcriptional level (1). Human GM-CSF gene transcription is regulated by various transcription factors whose binding sites have been identified in the most proximal promoter region upstream of the transcription start-point (2). These include NF-GMa and NF-GMb (3), NF-GM2 (NF-κB) (4,5), Elf-1 (6), NF-AT/AP-1 complex (7). The repeated sequence CATT(A/T) is required for inducible promoter activity in lymphoid T-cell lines and in primary lymphocytes (7–8). Recently, the transcription factors AP1, YY1, and an Sp1-related protein have been shown to be involved in regulation of the CATT enhancer activity (9).

HTLV-transactivator Tax can activate transcription indirectly by enhancing the activity of the transcription factors that recognize responsive elements located in the promoters of Tax-responsive genes. One class of target sites for Tax are the κB sequences which are bound by members of the Rel/NF-κB family. It has been previously shown that Tax is able to induce nuclear translocation of NF-κB (10) and that Tax activation on human GM-CSF requires the region including the NF-κB recognition site (11). A recent study has demonstrated that a distal regulatory element, located 3 kb upstream of the GM-CSF gene, functions as a strong cyclosporin A-sensitive enhancer (12). This suggests that important regulatory elements located far upstream of the transcription start point may significantly contribute to the transcriptional regulation of this gene.

We report here that an NF-κB like binding site, located between −2002 and −1984, acts as a positive transcriptional element in the Mo lymphoblastoid cell line, which constitutively produces GM-CSF (13).

MATERIALS AND METHODS

Plasmid constructions. Plasmid PF2000 (14), which contains the first 2010 bp upstream of the transcription start-site, was used to create, by means of PCR, three additional constructs lacking those DNA elements identified by DNase I footprinting. The oligonucleotide 5′-GCCATTGGGATATATCAACG-3′, complementary to a sequence in the 5′ end of the CAT gene,

[1] The first two authors contributed equally to this work.

[2] Present address: Department of Tumor Biology, The University of Texas M.D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030.

[3] To whom correspondence and reprint requests should be addressed. Fax: +39-10-3538978. E-mail: vanceci@igecuniv.csita.unige.it.

0006-291X/96 $18.00

was used as antisense primer for all constructs; the oligonucleotide 5′-TGTGAGGGCCAGTGGGAGGGTCCCGTTT-3′ corresponding to the −1908 to −1881 fragment (upper strand), was used to generate the pΔA construct. In the same way, the oligonucleotides 5′-GAACAGGTCCTGGTGTGGATTGGAAA-3′ (−1871 to −1846, upper strand) and 5′-GAGCTATGACAACTAATTTCTAGGAGGAAA-3′ (−1825 to −1796, upper strand), were used to generate respectively the pΔB and the pΔC constructs. After ligation with BamHI linkers, PCR products were digested with BamHI and BglII and subcloned in the polylinker of the pBLCAT 3 vector, upstream of the Chloramphenicol Acetyltransferase (CAT) gene. Plasmids PF24 and PF20 (14), containing respectively the first 240 and 1742 bp upstream of the transcription start site of the GM-CSF gene were also used.

Cell culture and transfection experiments. Mo cells were transfected by electroporation as follows: after washing twice with isotonic solution, 1.2×10^7 cells were resuspended in 0.5 ml of IMDM without serum and subjected to electroporation (200 Volts-960 mFa, Bio-Rad Gene Pulser TM), with 15 μg of CAT chimeric plasmid and 5 μg of pRSV-βgal. Following electroporation, cells were collected and cultured in complete medium with 10% FCS for about 60 h. 5637 cells were transfected by the calcium phosphate procedure (14). CAT activity was measured by standard methods (15) after normalizing for β-galactosidase activity and protein concentration. For all constructs, at least two different plasmid preparations were used. Relative CAT activities and SD were calculated from at least four experiments by using activity of the pPF24 set at a value of 100 as a reference.

DNase I footprinting. A 3′-end-labeled 298 bp footprinting probe, which includes the 269 bp promoter region from −1742 to −2010 and 29 bp of the vector polylinker, was prepared by cleaving plasmid pPF2000 with Hind III and by labeling at the 3′ end with Klenow enzyme and a dNTP mixture containing [α^{32}P]dCTP; Pvu II was used for the second digestion and the end-labeled DNA fragment was purified on a polyacrylamide gel. The DNA binding reaction was performed in a final volume of 50 μl containing 15 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, 0.5 mM dithiothreitol, 40 mM NaCl, 5 mM $MgCl_2$, 5 mM $CaCl_2$, 2.5 μg of poly (dI-dC), 25 fmol of the end-labeled DNA probe and nuclear extract (50 μg of protein), prepared according to the method of Dignam (16) or buffer used for dialysis of nuclear extracts. The binding reaction was allowed to proceed at room temperature for 15 min. DNase I (Boehringer, grade I, stored at −20° at a concentration of 1 mg/ml in 50% glycerol) was appropriately diluted (1:60 for samples containing nuclear extract and 1:800 for samples without extract) immediately before use in an ice cold buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 62.5 mM $MgCl_2$, 10 mM $CaCl_2$ and 1 mg/ml bovine serum albumin. Two μl were added to the binding mixture and incubated for 1 min (samples with extract) or 15 sec (samples without extract) at room temperature. The reaction was stopped with 15 μl of 5% SDS, 125 mM EDTA and 0.7 mg/ml tRNA, and the samples subjected to phenol-chloroform extraction and ethanol precipitation. The digestion products were then analyzed by electrophoresis on denaturing 8% polyacrylamide, 7 M urea gels. An aliquot of the same end-labeled DNA fragment was also subjected to the G + A sequencing reaction according to Maxam-Gilbert (17) and loaded on the same gel for identification of protected sequences.

Electrophoretic mobility shift assay (*EMSA*). Double-stranded oligonucleotides corresponding to the A element 5′-TCCTCAGCTCTGGACTTCCCCCT-3′ (upper strand) and 3′-GTCGAGACCTGAAGGGGGACGGCTCAG-5′ (lower strand), B element 5′-TGTGAGGGCCAGTGGGAGGGTCCCGTTT-3′ (upper strand) and 3′-GTCACCCTCC-CAGGGCAAATGGAG-5′ (lower strand) and to the NF-κB binding site of the human GM-CSF promoter 5′-CACAGTTCAGGTAGTTCCCCCGCCT-3′ (upper strand) and 3′-AAGTCCATCAAGGGGGCGGAGGGACCC-5′ (lower strand), were used as labeled probes or as unlabeled competitors. Blunt ended double stranded oligonucleotides described to bind selectively purified c-Rel (5′-GTACTAAAAAACCCGCTCGAGATCCTATG-3′, upper strand), purified p65 (5′-GTACCGGAAATTCCGGGCTCGAGATCCTATG-3′, upper strand), and purified p50 (5′-GTAGGGGGCCTCCCCGGCTCGAGATCCTATG-3′, upper strand) and the oligonucleotide containing the NF-κB motif present in the HIV-1 LTR (5′-GTAGGGGACTTTCCGAGCTCGAGATCCTATG-3′, upper strand) (18) were also used. Recessed 3′ ends were filled with Klenow enzyme by using a dNTP mixture containing [α^{32}P]dCTP and blunt ended oligonucleotides were labeled with T4-polynucleotide kinase and [γ^{32}P]ATP. Approximately 3 μg of nuclear extract were used in each binding reaction with 5 fmol of an end-labeled oligonucleotide (about 10,000 cpm), in a 10 μl incubation mixture containing 12.5 mM Hepes (pH 7.9), 100 mM NaCl, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol and 4 μg of poly (dI-dC) as a non-specific competitor. Samples were incubated for 20 min at room temperature and analyzed on a 5% polyacrylamide gel in 0.25 × TBE (22 mM Tris borate, 0.5 mM EDTA), and run at 150 volts for 2 h. Gels were dried prior to autoradiography at −80°C.

For supershift assay, the polyclonal antibody raised against the epitope corresponding to the amino acids 498–517 mapping within the carboxy terminal domain of mouse c-Rel (sc-71 Santa Cruz Biotechnology, Inc.), non-cross-reactive with related NF-κB p65 and Rel B p75, and a polyclonal antibody raised against a peptide corresponding to amino acids 3–19 mapping within the amino terminal region of human p65 (sc-109, Santa Cruz Biotechnology, Inc.) were used. The antibodies were added in the EMSA binding mixture after 10 min, at the amount of 0.1, 0.2, 0.5 and 1 μg; the mixture was then incubated for an additional 20 min at room temperature before gel electrophoresis.

Western blot. Mo nuclear extract (50 μg) was subjected to SDS-PAGE (10% polyacrylamide) and electrophoretically transferred to a nitrocellulose membrane (19). Blotting and immunodetection, using the same polyclonal antibody already employed for supershift assay, were performed as described (20).

RESULTS

Four Sequences between −1742 and −2010 in the Human GM-CSF Promoter Are Recognized by Nuclear Factors

We previously found that an upstream region between −1742 and −2010 in the human GM-CSF promoter has a positive role in the control of transcription (14). To analyze in detail this upstream promoter sequence, we looked for binding sites recognized by nuclear factors present in a cell line constitutively expressing GM-CSF, the T-lymphoid Mo. Footprinting experiments showed that four DNA elements were protected from DNase I digestion by proteins present in Mo cell nuclear extract. We defined them as A element, from −2002 to −1984; B element, from −1901 to −1887; C element, from −1864 to −1841; D element, from −1815 to −1791 (Fig 1, lane 3 and Fig 2).

Element A Acts as a Positive Promoter Element

In order to verify the role of the elements identified by DNase I footprinting assay on the transcriptional activity of the GM-CSF promoter, chimeric plasmids containing fragments of various length of the −1742 to −2010 region were fused to the CAT reporter gene and tested in DNA transient transfection experiments in cell lines that constitutively produce GM-CSF: the T-lymphoid Mo, and the carcinoma cell-line 5637. Plasmid ΔA, in which the sequence between −1909 and −2010 was deleted, showed a 40-fold lower activity than pPF2000 in the Mo cell line and a 5-fold lower activity in 5637 (Fig. 3). A comparable reduction was detected with plasmid PF20, in which the 269 bp region between −2010 and −1742, containing the four A to D elements,

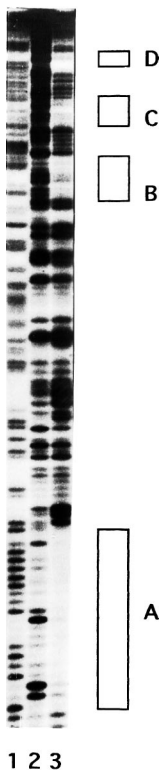


FIG. 1. DNase I footprints on a 3′ end-labeled segment of plasmid pPF 2000 containing the sequence between −1742 and −2010 of the GM-CSF promoter. Maxam-Gilbert G+A sequencing reaction (lane 1); without nuclear extract (lane 2); 50 μg Mo cell nuclear extract (lane 3).

FIG. 2. Sequences of GM-CSF promoter elements and oligonucleotides. (A) Sequences of protected elements in footprinting experiments. (B) Sequences of double-stranded oligonucleotides corresponding to the A element, B element and NF-κB binding site in GM-CSF promoter used in EMSA experiments. (C) Sequence comparison between the NF-κB sites in the A element, the human immunodeficiency virus long terminal repeat (HIV-LTR) and the Interleukin-2 receptor (IL-2R) "binding site."

was deleted. In all transfection experiments, the PF24 plasmid containing 240 bp upstream of the transcription start-site of the human GM-CSF gene was included and considered as 100%. In Mo cells CAT activity of pPF2000 was 4–5 fold higher than in 5637 cells (Fig 3). In previous experiments (14) with uninduced embryonal fibroblasts that do not express GM-CSF we observed no effect of deletion of the upstream (−2010 to −1742) region. These results indicate that the A element acts as a positive promoter element in two cell lines producing GM-CSF, although with quantitative differences.

An NF-κB/Rel Factor Binds an NF-κB-like Motif Contained in the A Element

To study in greater detail the properties of the nuclear factors that bind to the A element, we performed EMSA experiments with Mo nuclear extracts. Using as a labeled probe a double-stranded oligonucleotide containing the A element, a shifted DNA-protein complex was detected (Fig 4A, lane 1). This complex was specifically competed in a dose dependent manner by the same unlabeled oligonucleotide used as a probe (Fig 4A, lanes 2–5) and, although less efficiently, by a double stranded oligonucleotide containing the downstream NF-κB binding site between −76 and −85 (Fig 4A, lanes 6–9), whereas a double-stranded oligonucleotide containing the B element did not compete for the binding of the protein (Figure 4A, lanes 10–13). EMSA assays were then

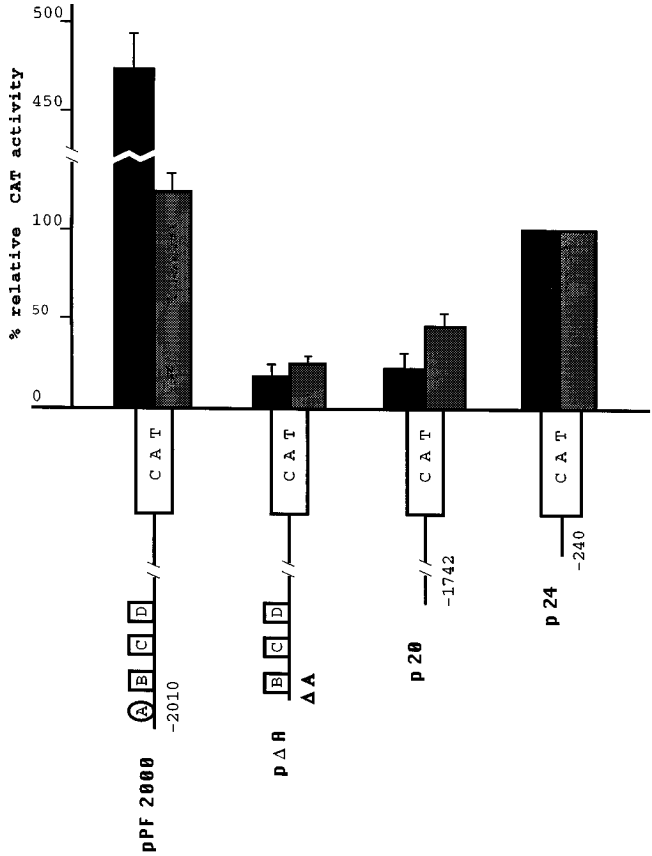
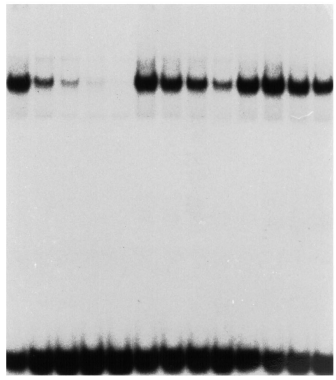


FIG. 3. Functional activity of the GM-CSF promoter constructs in Mo and 5637 cell lines. Results of CAT assays ±S.D. in Mo cell line (dark bars) and 5637 (grey bars). All transfections were repeated at least four times in duplicate.

performed with the A oligonucleotide as a labeled probe and different double stranded oligonucleotides as cold competitors: an oligonucleotide containing the NF-κB motif present in the HIV-1 LTR and oligonucleotides that were reported to bind selectively different members of the NF-κB/Rel family in a purified form (18). Competition with the HIV-1 NF-κB oligonucleotide was as efficient as competition with the A oligonucleotide itself, whereas oligonucleotides recognizing purified c-Rel (c-Rel oligonucleotide) and p65 (p65 oligonucleotide) competed with lower affinity and the oligonucleotide recognizing purified p50 (p50 oligonucleotide) did not compete (Fig 4B). Using the HIV-1 NF-κB oligonucleotide as a labeled probe with the same nuclear extract, we

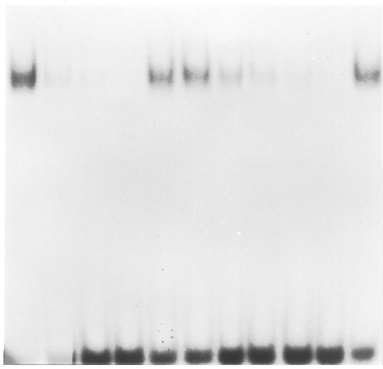
FIG. 4. Binding of a specific factor to a double-stranded oligonucleotide corresponding to the A element. (A) The labeled A oligonucleotide was incubated with 3 μg of crude nuclear extract prepared from the Mo lymphoblastoid T-cell line without competitor (lane 1); competition with 10, 25, 50, 100-fold molar excess of unlabeled A oligonucleotide (lanes 2–5), competition with 10, 25, 50, 100-fold molar excess of unlabeled oligonucleotide containing the GM-CSF NF-κB binding site (lanes 6–9); competition with 10, 25, 50, 100-fold molar excess of unlabeled B oligonucleotide (lanes 10–13). Excess of competitor oligonucleotides is referred to the labeled probe. (B) EMSA in the same conditions as in A, in the presence of different competitor oligonucleotides at 25, 50, 100 fold molar excess: no competitor (lanes 1, 11, 12, 22); A oligonucleotide (lanes 2, 3, 4 and 13, 14, 15); p65 oligonucleotide (lanes 5, 6, 7); HIV NF-κB oligonucleotide (lanes 8, 9, 10); c-Rel oligonucleotide (lanes 16, 17, 18); p50 oligonucleotide (lanes 19, 20, 21). (C) EMSA assay with Mo nuclear extract and A oligonucleotide (lanes 1 to 3) or p65 oligonucleotide (lanes 4 and 5) as labeled probes, in the absence of competitors (lanes 1, 3, 4) or in the presence of a 100 fold excess of unlabeled A oligonucleotide (lane 2) or p65 oligonucleotide (lane 5).

A

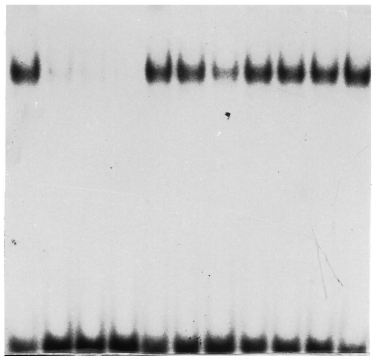


1 2 3 4 5 6 7 8 9 10 11 12 13

B

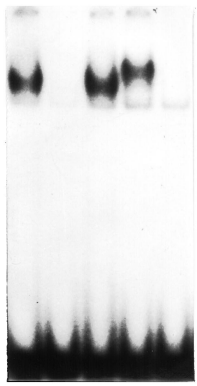


1 2 3 4 5 6 7 8 9 10 11



12 13 14 15 16 17 18 19 20 21 22

C



1 2 3 4 5

obtained a retarded complex identical, for electrophoretic mobility, to the one obtained with the A oligonucleotide (not shown) whereas the p65 oligonucleotide used as probe generated a retarded complex, presumably p65 homodimer, with lower mobility (Fig 4C).

Using a double stranded oligonucleotide containing the NF-κB binding site located between −76 and −85 as a labeled probe, two shifted DNA-protein complexes (I and II in Fig 5, lane 1) showed significant competitions with specific oligonucleotides: complex I was competed more efficiently by the oligonucleotide containing the A element (Fig 5, lanes 6–9) than by the oligonucleotide containing the NF-κB binding site (Fig 5, lanes 2–5). The opposite was observed with complex II, for which the NF-κB binding site was a better competitor (Fig 5, lanes 2–5) than the oligonucleotide containing the upstream A element (Fig 5, lanes 6–9). A double stranded oligonucleotide containing the B element did not compete for the two complexes I and II (Fig 5, lanes 10 and 11). This indicates that the promoter element between −76 and −85 is able to bind NF-κB (11) and also, with lower affinity, a factor that binds more efficiently to the upstream A binding site, possibly a different form of NF-κB. A comparison of the sequence corresponding to the A element, compared with binding sites of already characterized transcription factors (21), indicated that it is very similar to the DNA sequence recognized by c-Rel (Fig 2C). To test the hypothesis that the complex observed in EMSA experiments was due to the binding of a c-Rel containing factor, we performed an EMSA assay in the presence of a polyclonal antibody raised against the carboxyl terminal domain of mouse c-Rel (epitope corresponding to aminoacids 498–517) that is declared to be not cross-reactive with the related Rel A (p65) and Rel B (p75) proteins (Santa Cruz Biotechnology, technical notes). In an EMSA with the oligonucleotide containing the upstream A element as a labeled probe and Mo cell nuclear extract, the antibody caused, in a dose dependent manner, the reduction of intensity of the major retarded complex and the formation of a supershifted band (Figure 6, lanes 3–6) that was not detectable in the presence of preimmune serum (lane 1) and was competed by the specific A oligonucleotide as well as the major retarded complex (lane 7), thus indicating that c-Rel is a component of the DNA-protein complex. An EMSA experiment with an anti-p65 polyclonal antibody (Santa Cruz Biotechnology) did not show any effect on the retarded complex (data not shown). In a Western blot experiment using nuclear extract prepared from Mo cells and the anti-c-Rel polyclonal antibody, we found a band of 82 kDa corresponding to the expected c-Rel molecular weight (Fig 7).

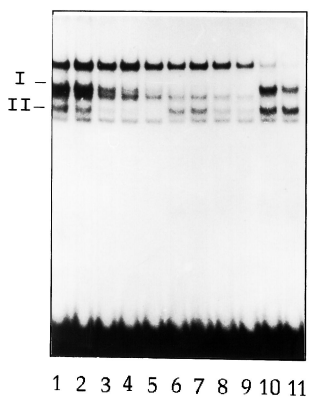


FIG. 5. Binding of specific factor to a double stranded oligonucleotide containing the NF-κB binding site in the human GM-CSF promoter (from −76 to −85). The labeled NF-κB oligonucleotide was incubated with 3 μg of Mo nuclear extract without competitor (lane 1); competition with 10, 25, 50, 100 fold molar excess of unlabeled NF-κB oligonucleotide (lanes 2 to 5); competition with 10, 25, 50, 100 fold molar excess of unlabeled A oligonucleotide (lanes 6 to 9); competition with 50, 100 fold molar excess of unlabeled B oligonucleotide (lanes 10 and 11). I and II indicate retarded complexes with significant competitions. Excess of competitor oligonucleotides is referred to the labeled probe.

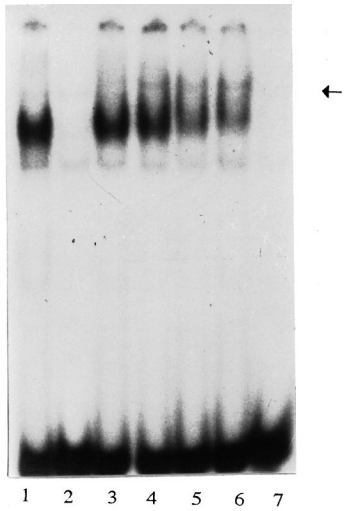


FIG. 6. EMSA assays in the presence of specific antibodies. The assay was performed with Mo nuclear extract (3μg) and A oligonucleotide as labeled probe in the presence of preimmune serum (lanes 1 and 2) or anti-c-Rel antibody at 0.1 (lane 3), 0.5 (lane 4), 1.0 (lanes 5 and 7) and 2.0 μg (lane 6); a 50 fold molar excess of A unlabeled oligonucleotide as competitor was included in lanes 2 and 7. The arrow indicates the supershifted band.

DISCUSSION

We report here that an upstream positive regulatory element in human GM-CSF promoter (the A element, from −1984 to −2002, containing the sequence 5′ GGGGAAGTCC 3′) binds an NF-κB factor, the composition of which, in term of active heterodimeric/homodimeric complex, is still to be clarified although our experiments suggest that c-Rel is involved. This finding is based on DNA/protein binding assays in which, using nuclear extracts from the lymphoblastoid Mo cell line, an anti-c-Rel antibody modified the pattern of retarded band, whereas an anti-p65 had no effect. We also show by competition with the specific oligonucleotides that presumably this same factor recognized, although with lower affinity, a downstream sequence (from −76 to −85, 5′ GGGAAC-TACC 3′) corresponding to the previously described NF-κB binding site (11).

The transfection experiments described here with cell lines that constitutively produce GM-CSF

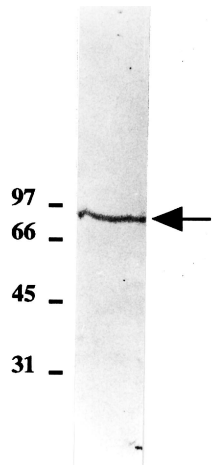


FIG. 7. Western blot analysis of nuclear extracts prepared from Mo cell line. Nuclear extract of the Mo cell line (50 μg) was fractionated by SDS-PAGE, blotted, reacted with anti-c-Rel specific antibody and immunostained. The position of molecular size markers is indicated in KDa.

and others previously described (14) with embryonal fibroblasts that, if uninduced, do not produce GM-CSF, indicate that the upstream element is relevant for constitutive expression. Constitutive GM-CSF production is particularly elevated in the T-lymphoid Mo cell line, derived from an atypical Hairy Cell Leukemia, infected by HTLV-II (22). Besides GM-CSF expression, this cell line displays several features of activated T-cells, such as expression of IL-2 receptor (23). The HTLV Tax transactivator has been reported to stimulate expression of c-rel (24, 25), suggesting the possibility that high expression of c-rel may contribute to the constitutive production of GM-CSF in the Mo cell line (26). T-cell specific Tax-responsive sequences have been previously localized in a 150 bp region extending from −114 to +37 in the human GM-CSF promoter by using HTLV-infected or Tax-transfected cell lines (11, 27, 28). This region contains the −76 to −85 NF-κB recognition site and a CK1 element that bind an NF-κB protein and CREB/ATF proteins (11).

Our results provide evidences that an upstream element in the 5′ flanking region of the GM-CSF gene is able to bind an NF-κB factor that positively regulates gene transcription and that c-Rel is likely to be a component of the NF-κB factor as confirmed by anti-c-Rel antibody recognition.

ACKNOWLEDGMENTS

We thank to Dr. M. Sirito, Dr. N. Bobola and Dr. S. Penco for technical assistance in some experiments, Prof. F. Ajmar for useful comments on the manuscript and Milena Ruscillo for her secretarial assistance. This work was supported in part by MURST 60% to G.B.-S., MURST 40% to C.G., CNR grants 93.01977.14 to G.B.-S., 94.00272.14 to C.G., A.I.R.C. to R.R. and AIRC 324/95 to G.B.-S.

REFERENCES

1. Gasson, J. C. (1991) *Blood* **77,** 1131–1145.
2. Fraser, J. K., Tran, S., Nimer, S. D., and Gasson, J. C. (1994) *Blood* **80,** 2523–2530.
3. Shannon, M. F., Gamble, J. R., and Vadas, M. A. (1988) *Proc. Natl. Acad. Sci.* **85,** 674–678.
4. Schreck, R., and Baeuerle, P. (1990) *Mol. Cell. Biol.* **10,** 1281–1286.
5. Tsuboi, A., Sugimoto, K., Yodoi, J., Miyatake, S., Arai, K., and Arai, N. (1991) *Int. Immunol.* **3,** 807–813.
6. Wang, C. Y., Bassuk, A. G., Boise, L. H., Thompson, C. B., Bravo, R., and Leiden, J. M. (1994) *Mol. Cell. Biol.* **14,** 1153–1159.
7. Masuda, E. S., Tokumitsu, H., Tsuboi, A., Shlomai, J., Hung, P., Arai, K., and Arai, N. (1993) *Mol. Cell. Biol.* **13,** 7399–7407.
8. Nimer, S., Fraser, J., Richards, J., Lynch, M., and Gasson, J. C. (1990) *Mol. Cell. Biol.* **10,** 6084–6088.
9. Ye, J., Zhanf, X., and Doug, Z. (1996) *Mol. Cell. Biol.* **16,** 157–167.
10. Munoz, E., and Israel, A. (1995) *Immunobiology* **193,** 128–136.
11. Himes, S. R., Coles, I. S., Katsikeros, R., Lang, R. K., and Shannon, M. F. (1993) *Oncogene* **8,** 3189–3197.
12. Cockerill, P. N., Bert, A. G., Jenkins, F., Ryan, G. R., Shannon, M. F., and Vadas, M. A. (1995) *Mol. Cell. Biol.* **15,** 2071–2079.
13. Wong, G. G., Witeck, J. S., Temple, P. A., Wilkens, K. M., Leary, A. C., Luxenburg, D. P., Jones, S. S., Brown, E. L., Kay, M. R., Orr, E. C., Shoemaker, C., Golde, D. W., Kaufman, R. J., Hewick, R. M., Wang, E. A., and Clark, S. C. (1985) *Science* **228,** 810–815.
14. Fiorentini, P., Musso, M., Penco, S., Giuffrida, R., Pistoia, V., Garré, C., and Bianchi-Scarrà, G. (1995) *Biochem. Biophys. Res. Commun.* **214,** 1015–1022.
15. Neumann, J. R., Morency, C. A., and Russian, K. O. (1987) *Bio Techniques* **5,** 444–447.
16. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) *Nucl. Aci. Res.* **11,** 1475–1489.
17. Maxam, A. M., and Gilbert, W. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 560–564.
18. Kunsch, C., Ruben, S. M., and Rosen, C. A. (1992) *Mol. Cell. Biol.* **12,** 4412–4421.
19. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci.* **76,** 4350–4354.
20. Kontgen, F., Grumont, R. J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D., and Gerondakis, S. (1995) *Genes Develop.* **9,** 1965–1977.
21. Faisst, S., and Meyer, S. (1992) *Nucl. Aci. Res.* **20,** 3–26.
22. Saxon, A., Stevens, R. H., and Golde, D. W. (1977) *Ann. Int. Med.* **16,** 777–781.
23. Depper, J. M., Leonard, W. J., Kronke, M., Walolmann, T. A., and Greene, W. C. (1985) *J. Immunol.* **133,** 1691–1695.
24. Arima, N., Molitor, J. A., Smith, M. R., Kim, J. H., Daitoku, Y., and Greene, W. C. (1991) *J. Virol.* **65,** 6892–6899.
25. Li, C., Ruscetti, F., Rice, N., Chen, E., Yang, N., Mikovits, J., and Longo, D. J. (1993) *Virol* **67,** 4205–4213.
26. Lal, L. B., Rudolph, D., Buckner, C., Pardi, D., and Hooper, C. (1993) *Blood* **81,** 1827–1832.
27. Nimer, S., Morita, E., Martis, M., Wachsman, W., and Gasson, J. C. (1988) *Mol. Cell. Biol.* **8,** 1979–1984.
28. Green, J., Begley, B., Wagner, D., Waldmann, T., and Jay, G. (1989) *Mol. Cell. Biol.* **9,** 4731–4737.