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Possible stage-specific function of NF-κB during pre-B cell differentiation

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Abstract Lipopolysaccharide (LPS)-induced differentiation of the murine pre-B cell line 70Z/3 is a model for pre-B to B cell differentiation and has been used to show that the transcription factor NF-κB is essential to induce the expression of the Igκ gene. We have investigated the mechanism involved in late stages of the process when all cells have reached a more mature B phenotype, i.e. beyond 48 up to 96 h of LPS treatment. NF-κB binding activity was induced at early times by LPS treatment, but its DNA binding activity disappeared after 84 h of LPS treatment. Accumulation of IκBα protein in the nucleus correlated with the disappearance of NF-κB activity at 72, 84 and 96 h, and treatment of nuclear extracts of 72–96 h LPS-treated cells with Na-deoxycholate restored NF-κB binding activity. The data indicate that NF-κB, while important to initiate the process of Igκ gene transcription in 70Z/3 pre-B cells, is no longer required for its maintenance in differentiated 70Z/3 cells.

Key words: B lymphocyte; Cellular differentiation; Transcription factor; Nuclear factor κB

1. Introduction

The pre-B 70Z/3 cell line represents a useful system for studying certain events of early B cell differentiation [1,2]. 70Z/3 cells contain a functionally rearranged but transcriptionally inactive Igκ locus. The activation of immunoglobulin transcription that occur in B cells is mediated by multiple cell-type-specific promoters and enhancers. Immunoglobulins promoters (μ, κ, λ) contain a highly conserved octanucleotide element essential for the expression of transfected immunoglobulin gene constructs in B cells [3,4]. The intronic κ enhancer also contains binding sites for the transcription factor NF-κB [5] required for B cell-specific demethylation and transcriptional activation of the locus [6,7]. NF-κB belongs to a family of transcription factors composed of five members (NFKB1, NFKB2, RelA, c-Rel and RelB), which share a 300 amino acid Rel homology domain and forming hetero- and homodimers [8]. NF-κB activity is regulated by specific inhibitors (IκB) that maintain the factor in a cytoplasmic, inactive form [9]. Several inducers cause dissociation and degradation of IκBs, and promote activation of NF-κB with a rapid translocation into the nucleus, where it directly regulates gene expression. While NF-κB factors are widely expressed in almost all cell types analysed so far, NF-κB has been reported to be constitutively activated only in mature B and T cells and in regions of the mouse brain [10–13]. The composition of NF-κB dimers is thought to change during the transition from pre-B to B cells [10,14,15]. On the other hand, resting fresh mouse spleen B cells do not contain NF-κB in the nucleus [16]. Others have reported that these cells contain NF-κB both in the nucleus and in the cytoplasm, with the cytoplasmic fraction being inducible by lipopolysaccharide (LPS) treatment [17]. Here we show that upon LPS treatment, 70Z/3 cells are induced to differentiate; a biphasic induction of Igκ and IκBα mRNA was observed, the initial phase correlated with nuclear translocation of NF-κB, while the second phase did not. At late time points of LPS treatment, NF-κB nuclear activity disappeared. Nuclear accumulation of IκBα correlated with down-regulation of NF-κB DNA binding activity. Nuclear extracts at this stage contained NF-κB binding activity which could be revealed only upon dissociation of NF-κB/IκB complexes. Thus, nuclear IκBα is likely the cause of NF-κB down-regulation. These data show that activation of NF-κB is required for the initiation of transcription of the Igκ gene in 70Z/3 pre-B cells, but not for its maintenance in differentiated 70Z/3 cells.

2. Materials and methods

2.1. Cell culture

HeLa and A549 cells were grown in DMEM medium supplemented with 10% calf serum, 0.01% penicillin-streptomycin and 2 mM L-glutamine. Murine pre-B cells 70Z/3 were grown in RPMI 1640 medium supplemented with 10% foetal calf serum, 0.01% penicillin-streptomycin, 2 mM L-glutamine with the addition of 50 μM 2-mercaptoethanol. 70Z/3 were stimulated with LPS (*Escherichia coli*) at a final concentration of 10 μg/ml [1].

2.2. Cytoplasmic and nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic extracts from cultured cells were prepared by standard procedures according to published methods [18]. The oligonucleotides used for EMSA were 5′-labelled with [γ-^{32}P]ATP, T4 polynucleotide kinase and were as follows:

κB: 5′-GCTGCCTGCTGGGGAAAGTAC-3′ [19];
Oct: 5′-CATGAATATGCAAATCAGGTGAGTCTAT-3′ [20].

Cytosolic extracts were treated with sodium desoxycholate for 10 min on ice to release NF-κB from IκB prior to the addition of the poly [d(I-C)], as previously described [21].

2.3. Western immunoblot analysis

10 μg of cytoplasmic and 5 μg of nuclear extracts were separated on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane (Bio-Rad). The blot was incubated with a 1:2000 dilution of the different antibodies (Santa Cruz Biotechnology, USA). The blot was developed according to the manufacturer's instructions (ECL, Amersham, UK).

2.4. Northern analysis

Total RNA was extracted from cells by the guanidinium isothiocyanate method [22]. 5 μg of total RNAs was loaded on a 1% agarose-formaldehyde gel and transferred to a nitrocellulose membrane (Bio-Rad). The following fragments were used as probes: a 0.317 kb frag-

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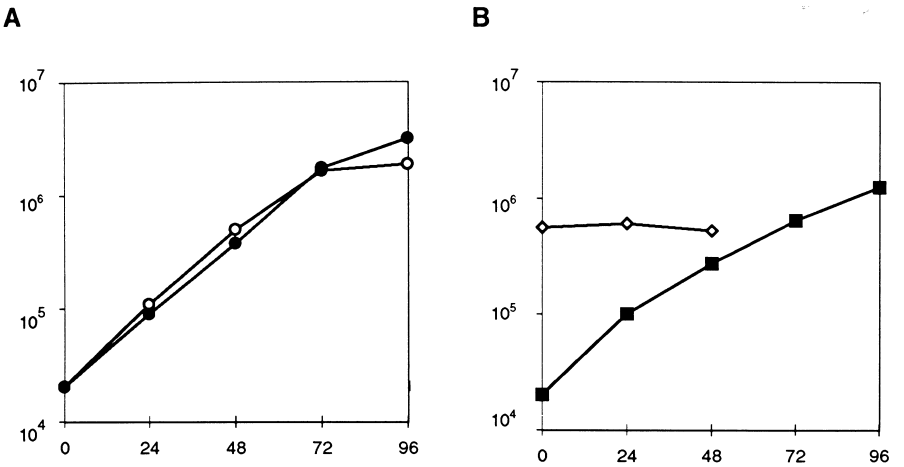

Fig. 1. Pre-B 70Z/3 cells differentiate upon LPS treatment. A: 70Z/3 cells were seeded at a starting cell density of 2×10^4 cells/ml. LPS was added 2 h after seeding. Vital counts of LPS-treated and untreated cells were taken every 24 h. ○, untreated cells; ●, LPS-treated cells. B: After 72 h of LPS treatment cells were diluted 1:3 in fresh medium containing no serum (final serum concentration 3.3%) and the growth rate of these cells was compared to untreated 70Z/3 cells grown in 3.3% serum. ◇, LPS-treated cells; ■, untreated cells.

ment from the human Igκ constant coding region was amplified by PCR from the plasmid p160 Mk6, with the following primers:

5′-CATTCCTGTTGAAGC-3′;
5′-GCTGATGCTGCACCA-3′.

The 1 kb IκBα cDNA was extracted from the RcCMV/MAD3 plasmid [21]. The rat GAPDH fragment [23] was used for normalisation of RNA loading. Fragments were labelled with [α-P^{32}]dCTP by the Random Primed DNA labelling kit (Boehringer). The intensity of RNA bands in the autoradiographs were quantitated by a video densitometer (Umax, Vista Speed).

3. Results

3.1. Pre-B 70Z/3 cells differentiate under prolonged LPS treatment

Previous studies with LPS-treated 70Z/3 cells were followed for no longer than 24/72 h [10,14]. In order to examine the maintenance of the differentiated state, we initially determined the optimal starting cell density that would allow at least 4 days (96 h) of LPS treatment with still vital and healthy cells. As shown in Fig. 1A, with a starting cell density of 2×10^4 cells/ml, the growth curves of untreated and LPS-treated 70Z/3 were essentially the same up to 72 h. The number of LPS-treated cells did not decrease up to 96 h, suggesting that no cell death was occurring under this culture conditions. Dilution of the cells in fresh medium after 72 h of LPS treatment did not stimulate further cell division, an indication of pre-B to B cell transition (Fig. 1B).

To verify the LPS-induced differentiation of 70Z/3 cells, we measured the expression of the Igκ mRNA and protein. Igκ mRNA (Fig. 2A) was induced after 24 h of LPS treatment (lane 4), remaining constant at 48 and 72 h (lanes 5 and 6). A further increase was observed at 84 and 96 h (lanes 7 and 8). The Igκ protein became detectable in the cytoplasmic extracts after 24 h of LPS (Fig. 2B, lane 5). These data indicate that pre-B 70Z/3 cells differentiate to mature IgM-secreting B cells under our culture conditions. We then used these cultures conditions for the subsequent experiments.

3.2. NF-κB is down-regulated at late time points of LPS treatment

In order to investigate the activation state of NF-κB and Oct-2 transcription factors in 70Z/3 cells upon LPS treatment, we prepared nuclear extracts from untreated and LPS-treated

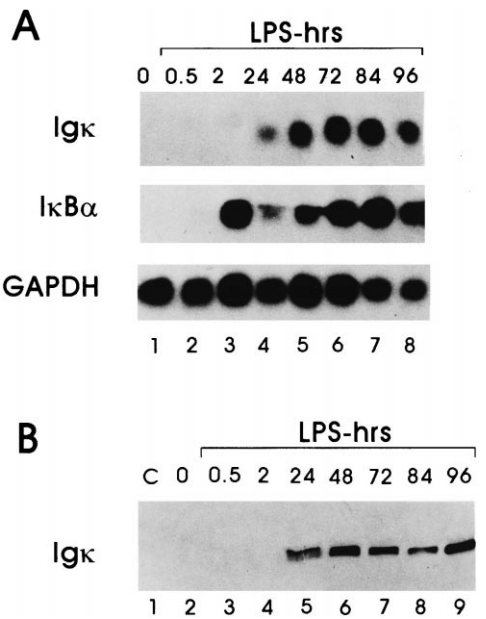

Fig. 2. Induction of Igκ mRNA and protein upon LPS-induced differentiation. A: Northern analysis of total mRNA extracted from 70Z/3 cells. Igκ mRNA was not detected in untreated cells (lane 1) or in cells treated for 0.5 and 2 h with LPS (lanes 2 and 3). After 24 h Igκ mRNA was detected (lane 4), it remained constant at 48 and 72 h (lanes 5 and 6) and increased at 84 and 96 h (lanes 7 and 8). IκBα mRNA expression was induced upon LPS treatment after 2 h (lane 3). At 84 h (lane 8) increased again. As control for RNA loading the filter was hybridised with a GAPDH probe. B: Immunoblot analysis of 70Z/3 cytoplasmic extracts. Igκ protein was not detected in untreated cells (lane 2) and in cells treated for 0.5 and 2 h with LPS (lanes 3 and 4). After 24 h Igκ protein was detected (lane 5) increasing at 48 and 72 h (lanes 6 and 7). Further increase was observed at 96 h (lane 9). Lane 1: HeLa cytoplasmic extracts were used as negative control.

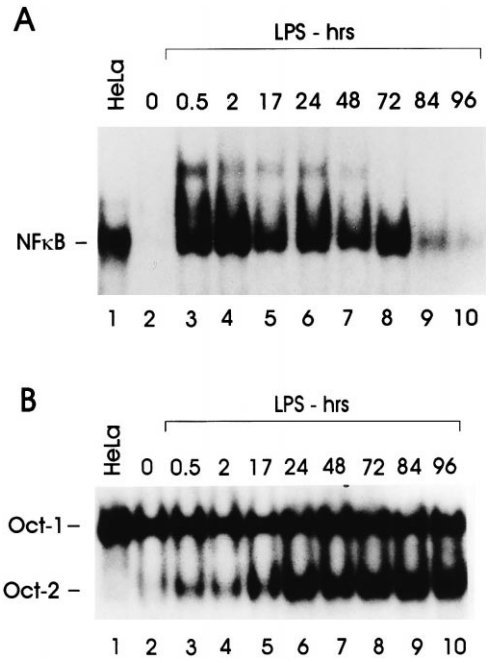

Fig. 3. NF-κB is down-regulated at late time points of LPS treatment. A: Nuclear extracts of 70Z/3 cells were analysed for NF-κB binding activity to an oligonucleotide reproducing the double HIV NF-κB site and to an oligonucleotide reproducing the Oct site from the VH promoter region. Untreated cells had no NF-κB binding activity (lane 2), but after 0.5 h of LPS NF-κB activity was induced (lane 3), and it remained constant up to 48 h (lanes 4–7). After 84 h NF-κB binding activity started to decline (lane 9), and at 96 h was absent as in untreated cells (lane 10). Oct-1 binding activity was not influenced by LPS treatment (lanes 2–10). Oct-2 was sligthly detectable in untreated cells (lane 2) but was rapidly induced by LPS (lanes 3–10). Lane 1: nuclear extracts from HeLa cells were used as control.

cells, and performed electrophoretic mobility shift assay (EMSA). As shown in Fig. 3, NF-κB binding activity was absent in untreated cells (lane 2), but was induced after 0.5 h of LPS treatment (lane 3) and remained activated up to 72 h (lanes 4–8), then steadily declined afterwards and was essentially absent at 96 h of treatment (lane 10). Oct-1 binding activity was not affected by the LPS treatment (lanes 2–10) while Oct-2, not present in untreated cells (lane 2), was induced early in the process and remained high throughout the 96 h of the experiment (lanes 3–10).

These data indicate a temporal discrepancy between Igκ expression and NF-κB activation at late stages of LPS treatment.

3.3. *NF-κB down-regulation at late time points of LPS treatments is not due to NF-κB subunit degradation*

To verify if NF-κB down-regulation at late time points of LPS treatments could be due to NF-κB subunit degradation, we used cytoplasmic and nuclear extracts from LPS-treated cells in immunoblot experiments. The RelA subunit remained constant in both the cytoplasm and nuclei for the 96 h of the experiment (Fig. 4). c-Rel, NFKB1 and NFKB2 were induced in both the cytoplasm and nuclei shortly after LPS addition. RelB was induced last, after 24 h of LPS treatment.

The IκBβ protein decreased in the cytoplasm after 0.5 h of LPS treatment (Fig. 4, lane 2), being completely absent after 2 h (lane 3). After 24 h de novo synthesised IκBβ protein became visible and remained constant thereafter (lanes 4–8). In the nuclear extracts we could not detect the IκBβ protein. On the other hand, LPS treatment of 70Z/3 cells did not result in IκBα protein degradation in either cytoplasm or nuclei. An accumulation of IκBα is visible in the nuclear extracts at 72, 84 and 96 h (lanes 14, 15 and 16). Northern analysis of RNA extracted from 70Z/3 cells revealed an initial increase of IκBα mRNA after 2 h of LPS, but after 72 h of LPS treatment, IκBα mRNA levels increased again (Fig. 2A).

The presence of all NF-κB subunits and inhibitors both in cytoplasmic and nuclear extracts at late LPS time points demonstrate that mechanisms other than NF-κB subunits degra-

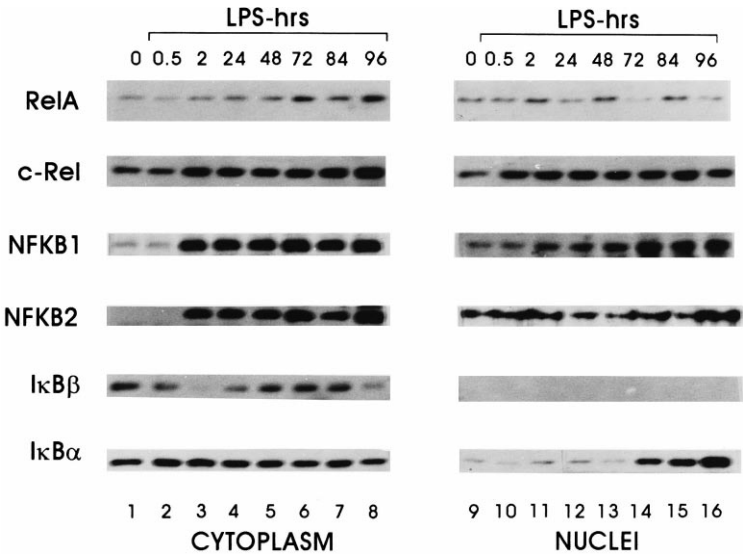

Fig. 4. Down-regulation of NF-κB DNA binding activity is not due to NF-κB subunit degradation. Immunoblot analysis of NF-κB subunits of 70Z/3 cells cytoplasmic and nuclear extracts. All subunits were present in both the cytoplasm and nuclear extracts of untreated cells (lanes 1 and 9, respectively). RelA expression was not induced by LPS, while c-Rel, NFKB1, NFKB2 and RelB were induced with different kinetics. IκBβ decreased after 0.5 h of LPS (lane 2) and after 2 h it was completely degraded (lane 3). After 24 h, IκBβ was visible again and remained constant thereafter (lanes 4–8). IκBα was not degraded in the cytoplasm upon LPS treatment (lanes 1–8); in the nuclear extracts an increase of the IκBα protein was detected after 72, 84 and 96 h of LPS (lanes 14, 15 and 16).

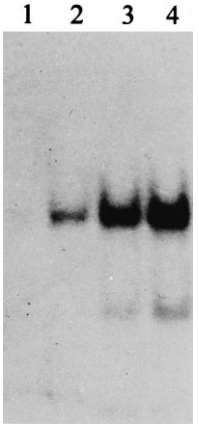

Fig. 5. NF-κB DNA binding activity is restored by deoxycholate treatment. Nuclear extracts of untreated cells (lane 1) and cells treated for 72, 84 and 96 h with LPS (lanes 2, 3 and 4) were treated with detergents in order to release the IκB-bound NF-κB and analysed for NF-κB binding activity to an oligonucleotide reproducing the double HIV NF-κB site.

dation, must be involved in down-regulation of NF-κB DNA binding activity.

3.4. *Nuclear accumulation of IκBα could account for down-regulation of NF-κB DNA binding activity*

The timing of IκBα protein accumulation in the nuclear extracts at 72–96 h correlates with down regulation of NF-κB DNA binding activity. In order to verify if nuclear accumulation of IκBα after 72 h of LPS treatment was involved in the decrease of NF-κB binding activity, nuclear extracts of untreated and LPS-treated cells for 72, 84 and 96 h were activated with the detergent deoxycholate in order to release the IκB-bound NF-κB and used in EMSA. An increase in detergent-activated NF-κB binding activity was observed (Fig. 5, lanes 2, 3 and 4).

These data suggest that de novo synthesised nuclear IκBα could be responsible for decreased NF-κB binding activity at late time points of LPS treatment.

4. Discussion

Previous studies have shown that NF-κB complexes are retained in the cytoplasm of pre-B cell lines, while in B cell lines NF-κB complexes are constitutively nuclear with developmental stage-specific changes in subunits composition [10,14]. 70Z/3 cells have been widely used as model of B cell differentiation, since treatment with LPS leads to differentiation towards a more mature B cell phenotype. This process has been thoroughly studied, but in all cases the time of induction has been limited to the first 72 h. We have determined conditions to extend these studies beyond this time (Fig. 1), and have therefore analysed this process up to 96 h, a time in which cells may have gone beyond the pre-B cell stage, since they do produce Igκ (Fig. 2) and have lost the ability to reproduce (Fig. 1B). Under these conditions, we found that no active NF-κB was present in nuclei at late time points of LPS treatment (Fig. 3), which implies that this factor is not required for the maintenance of this phenotype. On the other hand, the ubiquitous Oct-1 factor is not affected by the LPS treatment, while the B cell-specific factor Oct-2 is induced at early times by LPS and remains high through the 96 h (Fig. 3). Thus this factor may be involved in κ gene expression. This would be in agreement with previous studies with 70Z/3 cells, where TGF-β treatment of LPS-stimulated cells blocked both κ gene and Oct-2 expression but not NF-κB activation, demonstrating that NF-κB by itself is necessary but not sufficient to induce κ gene expression [24]. Our failure to detect NF-κB in mature 70Z/3 cells is consistent with the finding that primary splenic mouse B cells lack constitutive NF-κB binding activity [25], suggesting that the role of NF-κB in B cells could be important for developmental cellular processes but not for the maintenance of the differentiated phenotype.

The presence of all NF-κB subunits at late LPS time points demonstrates that mechanisms other than NF-κB subunits degradation must be involved in down-regulation of NF-κB DNA binding activity (Fig. 4). The absence of nuclear NF-κB DNA binding activity at late LPS time points appears to be due to an intranuclear inhibition, since treatment of nuclear extracts with sodium deoxycholate restores the DNA binding activity (Fig. 5). While we cannot exclude that other inhibitors are involved in this process, our data suggest that nuclear IκBα is responsible for inhibition of NF-κB DNA binding activity. Moreover, we could not find evidence of the presence of IκBβ in the nucleus, while we could easily detect it in the cytoplasm. Indeed, we found an increase of IκBα protein (Fig. 4) in the nucleus. Evidence for a role of the nuclear export sequences present in the C-terminal domain of IκBα in the export of NF-κB from the nucleus has also been recently provided [26,27]. Together these data converge to indicate that following its nuclear translocation, IκBα releases NF-κB from its target sites and consequently down-regulates NF-κB-dependent transcription.

The B cell-specific transcription factor Oct-2, almost absent in unstimulated 70Z/3 cells, rapidly increased upon LPS treatment and remained high for the whole 96 h of the experiment. Oct-2, rather than NF-κB, seems to be crucial for B cell terminal differentiation. NFKB1, c-Rel and RelB knock-out mice do not display a defect in B lymphocyte differentiation [28–30], although in the various knock-out animals other members of the NF-κB/Rel family might substitute for one another producing no major phenotypic alteration. In fact, NF-κB appears to be essential in B cell maturation, as double recombinant mice lacking both NFKB1 and NFKB2 genes did not produce mature B cells [31]. On the other hand, Oct-2 knock-out mice contain normal numbers of B cell precursors but are somehow deficient in IgM^+ B cells [32]. Oct-2 is not required for the generation of immunoglobulin-bearing B cells but is crucial for their maturation to immunoglobulin secreting cells. Thus Oct-2 induction may be essential to establish and maintain κ gene transcription in mature B cells.

NF-κB and Oct-2 may act in a combinatorial manner to initiate the stage-specific expression of the κ locus. According to this model, in the pre-B cell, NF-κB is cytoplasmically localised in an inactive complex with the inhibitors IκBα and IκBβ. The Oct-2 protein is present at low levels and unable to stimulate κ gene transcription in the absence of NF-κB. A differentiation signal like LPS results in rapid dissociation of NF-κB from IκBβ and translocation of the former to the nucleus. Upon binding, the κB motif in the κ gene intron enhancer, NF-κB, in concert with the pre-existing Oct-2 protein, may stimulate low-level κ gene transcription.

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References

[1] Paige, C., Kincade, P. and Ralph, P. (1978) J. Immunol. 121, 641–647.
[2] Mains, P.E. and Sibley, C.H. (1983) J. Biol. Chem. 258, 5027–5033.
[3] Falkner, F.G. and Zachau, H.Z. (1984) Nature 310, 71–74.
[4] Currie, R. and Roeder, R. (1989) Mol. Cell. Biol. 9, 4239–4247.
[5] Sen, R. and Baltimore, D. (1986) Cell 46, 705–716.
[6] Kirillov, A., Kistler, B., Motoslavsky, R., Cedar, H., Wirth, T. and Bergman, Y. (1996) Nature Genet. 13, 435–441.
[7] Lenardo, M., Pierce, J. and Baltimore, D. (1987) Science 236, 1573–1577.
[8] Baeuerle, P.A. (1991) Biochim. Biophys. Acta 1072, 63–80.
[9] Verma, I., Stevenson, J., Schwarz, E., Antwerp, D. and Miyamoto, S. (1995) Genes Dev. 9, 2723–2735.
[10] Miyamoto, S., Schmit, M.J. and Verma, I. (1994) Proc. Natl. Acad. Sci. USA 91, 5056–5060.
[11] Schmidt-Ullrich, R., Memet, S., Liliebaum, A., Feuillard, J., Raphael, M. and Israel, A. (1996) Development 122, 2117–2128.
[12] Guerrini, L., Blasi, F. and Denis-Donini, S. (1995) Proc. Natl. Acad. Sci. USA 92, 9077–9081.
[13] Guerrini, L., Molteni, A., Wirth, T., Kistler, B. and Blasi, F. (1997) J. Neurosci. 17, 6057–6063.
[14] Liou, H., Sha, W., Scott, M. and Baltimore, D. (1994) Mol. Cell. Biol. 14, 5349–5359.
[15] Grumont, R.J. and Gerondakis, S. (1994) Cell Growth Different. 5, 1321–1331.
[16] Liu, J.L., Chiles, T.C., Sen, R.J. and Rothstein, T.L. (1991) J. Immunol. 146, 1685–1691.
[17] Marcuzzi, A., Van Ness, B., Rouse, T. and Lafrenz, D. (1989) Nucleic Acids Res. 17, 10455–10472.
[18] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[19] Nabel, G. and Baltimore, D. (1987) Nature 326, 711–713.
[20] Yang, Y.S., Hanke, J.H., Carayannopoullos, L., Craft, C.M., Capra, J.D. and Tucker, P.W. (1993) Mol. Cell. Biol. 13, 5593–5603.
[21] Hansen, S.K., Nerlov, C., Zabel, U., Verde, P., Johnsen, M., Baeuerle, P.A. and Blasi, F. (1992) EMBO J. 11, 205–213.
[22] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
[23] Piechaczyk, M.J., Blanchard, L., Marty, C., Dani, F., Panabieres, S.E., Fort, P. and Jeanteur, P. (1984) Nucleic Acids Res. 12, 6951–6963.
[24] Briskin, M., Kuwabara, M.D., Sigman, D.S. and Wall, R. (1988) Science 242, 1036–1037.
[25] Yedidia, Y., Ben-Neriah, Y. and Jung, S. (1998) Eur. J. Immunol. 28, 30–36.
[26] Arenzana-Seisdedos, F., Thompson, J., Rodriguez, M., Bachelerie, F., Thomas, D. and Hay, R. (1995) Mol. Cell. Biol. 15, 2689–2696.
[27] Arenzana-Seisdedos, F., Turpin, P., Rodriguez, M., Hay, R.T., Vireliez, J.L. and Dargemont, C. (1997) J. Cell Sci. 110, 369–378.
[28] Sha, W., Liou, H., Tuomanen, E. and Baltimore, D. (1995) Cell 80, 321–330.
[29] Kontgen, F., Grumont, R.J., Strasser, A., Metcalf, D., Li, R., Tarlinton, D. and Gerondakis, S. (1995) Genes Dev. 9, 1965–1977.
[30] Weih, F., Carrasco, D., Durham, S., Barton, D., Rizzo, C., Ryseck, R., Lira, S. and Bravo, R. (1995) Cell 80, 331–340.
[31] Franzoso, G., Carlson, L., Xing, L.P., Poliak, L., Shores, E.W., Brown, K.D., Leonardi, A., Tran, T., Boyce, B.F. and Siebenlist, U. (1997) Genes Dev. 11, 3482–3496.
[32] Corcoran, L., Karvelas, M., Nossal, G., Ye, Z.S., Jacks, T. and Baltimore, D. (1993) Genes Dev. 7, 570–582.