A20, an inhibitor of cell death, self-associates by its zinc finger domain

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Abstract A20 is a primary response gene which is induced after monocyte adherence or cytokine stimulation of a variety of cells. The A20 protein belongs to a novel class of Cys_2/Cys_2 zinc finger proteins, and has been characterized as an inhibitor of both apoptotic and necrotic cell death. In order to clarify its molecular mechanism of action, we used the yeast-based two-hybrid system to screen for A20-associated proteins. Here we report that A20 is able to self-associate, and demonstrate that the latter interaction is mediated by its zinc finger domain.

Key words: Zinc finger protein; Protein–protein interaction; Self-association; Apoptosis; Necrosis; Tumor necrosis factor

1. Introduction

A20 is a primary response gene which was originally identified as a cytokine-inducible gene in human umbilical vein endothelial cells [1]. Subsequent research demonstrated that A20 is not only induced in endothelial cells by bacterial lipopolysaccharide or the cytokines tumor necrosis factor (TNF) and interleukin-1, but also by other stimuli in other cell types. These include the Epstein-Barr virus LMP1 gene product [2] and the B-cell surface receptor CD40 [3] in B-cells, as well as the human T-cell leukemia virus type I Tax and phorbol 12-myristate 13-acetate [4] in Jurkat cells. Superinduction of the A20 gene can often be observed by costimulation with cycloheximide. Furthermore, A20 is constitutively expressed in some cell lines resistant to TNF-mediated cytotoxicity [5], suggesting a role for A20 in promoting resistance to TNF-induced cell death. This hypothesis was further supported by the observation that introduction of the A20 gene in fibroblast cell lines susceptible to TNF-mediated cytotoxicity rendered the cells resistant to TNF-induced apoptosis [5] or TNF-induced necrosis (K. Heyninck, unpublished observations). Remarkably, the same gene has been cloned as a monocyte adherence cDNA clone, and is therefore also called MAD6 [6].

The molecular mechanism of action responsible for A20-induced cellular resistance to various forms of cell death is at present unknown. The human A20 gene encodes a 790 amino acid-containing protein, consisting of a C-terminal domain with seven Cys_2/Cys_2 zinc fingers [7]. In accordance with several known zinc finger proteins (reviewed in [8]), A20 has been postulated to function as a transcriptional activator [7], but specific DNA binding has never been demonstrated. Here we show that A20 is able to self-associate by its zinc finger domain, pointing at a role for the seven Cys_2/Cys_2 zinc fingers in protein–protein interaction.

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2. Materials and methods

2.1. Materials

The yeast expression vectors pAS2 and pGAD424, which encode the yeast Gal4 DNA-binding domain (Gal4DB) and the yeast Gal4 activation domain (Gal4AD), respectively, as well as the *Saccharomyces cerevisiae* HF7c reporter host strain are contained in the Matchmaker two-hybrid system from Clontech Laboratories (Palo Alto, CA, USA). Polyclonal antibodies recognizing Gal4DB were obtained from Upstate Biotechnology (Lake Placid, NY, USA). Alkaline phosphatase-conjugated goat anti-rabbit antibodies, 3-amino-1,2,4-triazole, 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium were from Sigma Chemical Co. (St. Louis, MO, USA). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside was from Saxon Biochemicals (Hannover, Germany).

2.2. Reverse transcriptase PCR cloning of human A20

Poly(A)$^+$ RNA was isolated from MCF7 human breast carcinoma cells which had been treated for 4 h with recombinant human TNF (1000 IU/ml) and cycloheximide (10 μg/ml). Using 0.5 μg of poly(A)$^+$ RNA, first-strand cDNA synthesis was catalyzed by Moloney murine leukemia virus reverse transcriptase, with a combination of an oligo-dT_{15} primer and a specific antisense primer starting at position 3351 in the 3′-untranslated region (5′-AGCTCATGCCCCAACAACAA-3′). After RNase H treatment, two overlapping fragments of A20 were amplified in a PCR reaction by *Pyrococcus furiosus* DNA polymerase. The primer pairs were 5′-GGTATACATATGGCTGAACAAGTCCTTCC-3′/5′-TGCTCGTCCCCGTCCTGTCC-3′ and 5′-CGCCCAACTGCCCCTTCTTC-3′/5′-AGCTCATGCCCCAACAACAA-3′. For subsequent cloning purposes an *Nde*I site was inserted at the start codon in the first primer. The two PCR-amplified cDNAs (67–1882 and 1232–3351) were digested by *Bam*HI and ligated at position 1614. The whole gene was subsequently cloned as an *Nde*I-*Xho*I (67–2478) fragment into the prokaryotic expression vector pLT10T3 [9].

2.3. Construction of two-hybrid fusion vectors

Gal4DB-A20 and Gal4AD-A20 fusion vectors were constructed by in-frame cloning into the yeast expression vectors pAS2 and pGAD424, respectively (Fig. 1). The full-length A20 cDNA was cloned into vector pAS2 as an *Nde*I-*Sal*I fragment (hereafter pAS2A20), and into vector pGAD424 as an *Eco*RI-*Sal*I fragment of pAS2A20 (hereafter pGAD424A20).

The C-terminal-deleted A20 cDNA, spanning amino acids 1–367 and lacking the seven Cys_2/Cys_2 zinc fingers, was cloned into vector pAS2 as an *Nde*I-blunted Bsp1286I fragment (hereafter pAS2A20ZF$^-$), and into vector pGAD424 as an *Eco*RI-*Sal*I fragment of pAS2A20ZF$^-$ (hereafter pGAD424A20ZF$^-$).

The N-terminal-deleted A20 cDNA, spanning amino acids 373–790 and containing the seven Cys_2/Cys_2 zinc fingers, was cloned into vector pAS2 as an *Nco*I-*Xho*I fragment (hereafter pAS2A20ZF$^+$), and into vector pGAD424, which was previously modified by cloning an *Nde*I linker with in-frame *Nco*I site (hereafter pGAD424A20ZF$^+$).

2.4. Construction of the L929r2 cDNA library

Poly(A)$^+$ RNA was isolated from L929r2 murine fibrosarcoma cells [10] which had been treated for 4 h with recombinant murine TNF (1000 IU/ml) and cycloheximide (10 μg/ml). First-strand cDNA synthesis was catalyzed by Superscript II reverse transcriptase either with oligo-dT_{15} primers or random primers. The blunted cDNA was ligated to *Eco*RI adapters and cloned into the pGAD424 yeast expression vector. The average insert length was 1400 base pairs. For library

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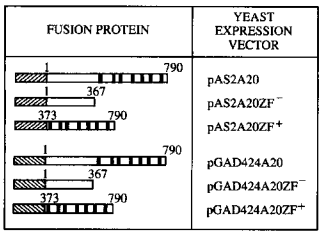

Fig. 1. Schematic representation of Gal4–A20 fusion proteins encoded by the respective yeast two-hybrid expression vectors. Hatched boxes represent the Gal4DB region (upper three) and the Gal4AD region (lower three). Numbers show the in-frame cloned A20 fragments. Black segments represent the seven Cys_2/Cys_2 zinc fingers within A20 cDNA.

screening, $>10^6$ individual cDNA inserts were used after pooling of both L929r2 library types.

2.5. Yeast-based two-hybrid screening

The yeast-based two-hybrid screening was essentially performed as described in the manufacturer's directions. Transformation of Gal4–A20 fusion vectors in the *S. cerevisiae* strain HF7c was achieved with the lithium acetate method [11]. Transformed cells were grown on appropriate synthetic minimal media using Trp1 and Leu2 transformation markers. Yeast colonies carrying putative interacting proteins were selected by growth on synthetic minimal media lacking Trp, Leu and His in the presence of 5 mM 3-amino-1,2,4-triazole, and by screening for β-galactosidase (βGal) activity in a filter assay using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as a substrate. Positively scoring pGAD424 vectors from the L929r2 cDNA library were recovered after electroporation of the plasmid mixtures into the LeuB *Escherichia coli* strain HB101 and selection on M9 media lacking Leu.

cDNA inserts encoding candidate A20-associating protein fragments were sequenced on both strands with a cycle sequencer (Applied Biosystems, Foster City, CA, USA). Network BLAST searches were conducted using the NCBI on-line service.

2.6. Detection of Gal4DB–A20 fusion proteins by Western blotting

Transformation of pAS2A20, pAS2A20ZF$^-$ and pAS2A20ZF$^+$ was performed in the *S. cerevisiae* strain HF7c using the lithium acetate method [11]. After growth on synthetic minimal medium lacking Trp, single colonies were inoculated in 5 ml of the same medium. At $A_{600} = 1.5$, the yeast protein fraction of the entire culture was separated by 10% SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane (Schleicher and Schuell, Dassel, Germany). Detection of the expressed Gal4DB-A20 fusion proteins was performed using a polyclonal anti-yeast Gal4DB antibody and an alkaline phosphatase-conjugated goat anti-rabbit antibody, with nitroblue tetrazolium plus 5-bromo-4-chloro-3-indolyl phosphate as a substrate.

2.7. Detection of A20ZF$^+$ cDNA by Southern blotting

L929r2 cDNA inserts encoding candidate A20 interacting proteins were obtained by *Eco*RI digestion of positively scoring pGAD424 vectors from the L929r2 cDNA library, followed by electrophoresis on 1% agarose gel. The DNA was transferred onto Hybond-N nylon membrane (Amersham International, Amersham, UK) and hybridized with a ^{32}P-labeled probe corresponding to the human A20ZF$^+$ fragment (*Nco*I-*Xho*I). Hybridization was performed at 60°C in buffer containing 5×SSC, 5×Denhardt's solution, 0.5% SDS and 20 μg/ml herring sperm DNA. The blot was washed with 2×SSC–0.1% SDS at room temperature and subsequently at 60°C.

3. Results

3.1. Self-association of A20

Screening of a cDNA library for interacting proteins with the yeast two-hybrid system requires several initial control experiments. In one of these, we tested whether A20 is able to self-associate. To this end, the full-length A20 cDNA was fused to Gal4DB and Gal4AD in the yeast expression vectors pAS2 and pGAD424, respectively (Fig. 1). The *S. cerevisiae* strain HF7c, which contains the His3 and lacZ reporter genes under control of a Gal4 promoter, was cotransformed with the latter plasmids. Cotransformation of the yeast expression vectors pVA3 and pTD1, which encode the strong interacting proteins p53 and SV40 large T-antigen, respectively, served as a positive control. Transformed cells were tested for their ability to grow on His-deficient plates in the presence of 5 mM 3-amino-1,2,4-triazole and for the expression of βGal activity. Interestingly, the latter phenotype was also expressed by cells cotransformed with pAS2A20 and pGAD424A20, and not by cells which were only transformed with a single plasmid or with the empty expression vectors (Table 1). These results clearly indicate that A20 is able to self-associate. To establish that the self-interaction of A20 is specific, the ability of a non-relevant protein to interact with A20 was tested. Cotransformation of the *S. cerevisiae* strain HF7c with pAS2A20 and pTD1, or with pVA3 and pGAD424A20, did not result in His auxotrophy or βGal activity, demonstrating the specificity of self-association.

3.2. Self-association of A20 is mediated by the zinc finger domain

Since the human A20 cDNA encodes a 790 amino acid-containing protein consisting of seven Cys_2/Cys_2 zinc fingers in the C-terminal half of the molecule, we investigated whether self-association of A20 is mediated by this domain. To this end, C-terminal-deleted and N-terminal-deleted A20 cDNA fragments were fused to Gal4DB (pAS2A20ZF$^-$ and pAS2A20ZF$^+$, respectively) and Gal4AD (pGAD424A20ZF$^-$ and pGAD424A20ZF$^+$, respectively). As outlined in Fig. 1, A20ZF$^-$ spans amino acids 1–367, and A20ZF$^+$ amino acids 373–790. To ensure that fusion proteins were expressed by transformed HF7c cells, extracts were analyzed by Western blotting using polyclonal anti-yeast Gal4DB antibodies. The latter revealed differentially expressed, but clearly detectable, Gal4DB-A20, Gal4DB-A20ZF$^-$ and Gal4DB-A20ZF$^+$ fusion protein levels (Fig. 2). Expression of Gal4AD fusion proteins could not be tested because suitable anti-Gal4AD antibodies are not available.

After *S. cerevisiae* strain HF7c had been cotransformed with the yeast expression vectors encoding the A20 deletion mutant fusion proteins, the following protein–protein interac-

Table 1
Self-association of A20

Transformed yeast expression vector(s)		Phenotype	
		His$^+$	βGal$^+$
pAS2		–	–
pAS2A20		–	–
	pGAD424	–	–
	pGAD424A20	–	–
pAS2	pGAD424	–	–
pAS2	pGAD424A20	–	–
pAS2A20	pGAD424	–	–
pAS2A20	pGAD424A20	+	+
pVA3	pTD1	+	+
pAS2A20	pTD1	–	–
pVA3	pGAD424A20	–	–

Survey of His auxotrophy (tested in the presence of 5 mM 3-amino-1,2,4-triazole) and βGal activity after (co)transformation of *S. cerevisiae* strain HF7c with full-length A20 or control yeast two-hybrid expression vectors. (Co)transformation was verified by growth on appropriate minimal synthetic media.

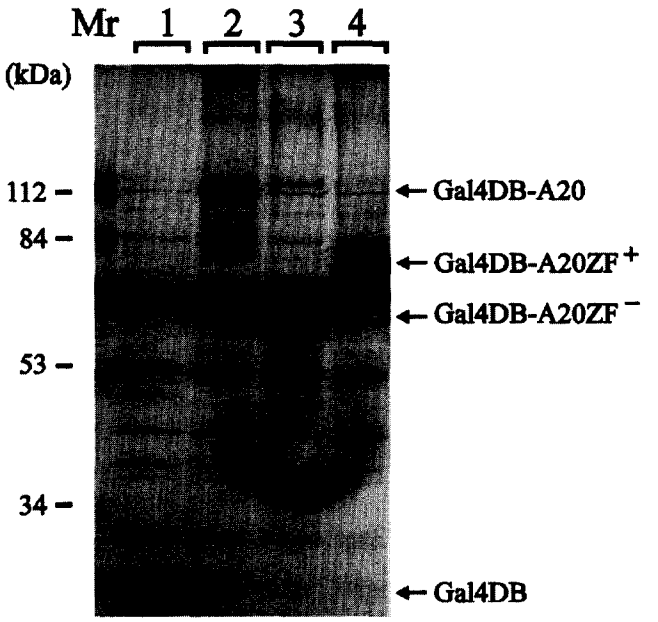

Fig. 2. Detection of Gal4DB–A20 fusion proteins by Western blotting. Protein fractions of *S. cerevisiae* strain HF7c, transformed with pAS2 (lane 1), pAS2A20 (lane 2), pAS2A20ZF$^-$ (lane 3) or pAS2A20ZF$^+$ (lane 4), were subjected to SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting using anti-Gal4DB antibodies.

tions were revealed by His auxotrophy and βGal activity (Table 2): (i) between Gal4DB-A20 and Gal4AD-A20, (ii) between Gal4DB-A20ZF$^+$ and Gal4AD-A20, and (iii) between Gal4DB-A20ZF$^+$ and Gal4AD-A20ZF$^+$. No protein–protein interaction with any Gal4-A20ZF$^-$ fusion protein was observed. Surprisingly, protein–protein interaction between Gal4DB-A20 and Gal4AD-A20ZF$^+$ could not be demonstrated, which possibly may be ascribed to steric hindrance between both fusion proteins under the in vivo conditions used. The observations strongly suggest that self-association of A20 is mediated by its zinc finger domain.

3.3. Screening of an L929r2 cDNA library for A20 interactions confirms association with the zinc finger domain of A20

After screening an L929r2 murine fibrosarcoma cDNA library with pAS2A20 as a bait, four cDNA inserts encoding candidate A20-associating protein fragments were retained.

Table 2
Self-association of A20 is mediated by its zinc finger domain

Transformed yeast expression vectors		Phenotype	
		His$^+$	βGal$^+$
pAS2A20	pGAD424A20	+	+
pAS2A20	pGAD424A20ZF$^-$	−	−
pAS2A20	pGAD424A20ZF$^+$	−	−
pAS2A20ZF$^-$	pGAD424A20	−	−
pAS2A20ZF$^+$	pGAD424A20	+	+
pAS2A20ZF$^-$	pGAD424A20ZF$^-$	−	−
pAS2A20ZF$^-$	pGAD424A20ZF$^+$	−	−
pAS2A20ZF$^+$	pGAD424A20ZF$^-$	−	−
pAS2A20ZF$^+$	pGAD424A20ZF$^+$	+	+

Survey of His auxotrophy (tested in the presence of 5 mM 3-amino-1,2,4-triazole) and βGal activity after cotransformation of *S. cerevisiae* strain HF7c with full-length, C-terminal-deleted or N-terminal-deleted A20 yeast two-hybrid expression vectors. Cotransformation was verified by growth on appropriate minimal synthetic media.

Southern blot analysis of these inserts was carried out using the human A20ZF$^+$ fragment as a probe. Indeed, one of these inserts hybridized to the A20ZF$^+$ probe, identifying it as an A20 fragment (Fig. 3, lane 2). DNA sequencing on both strands identified the hybridizing insert as a cDNA insert with an open reading frame encoding amino acids 399–775 of murine A20 [12]. This murine A20 fragment spans the second half of the first zinc finger followed by six entire zinc fingers, and is almost identical to the human A20ZF$^+$ fragment. The other three cDNA inserts showed no hybridization with the human A20ZF$^+$ fragment and displayed no sequence homology with murine A20. Their identity is being further investigated.

4. Discussion

Since the abundant *Xenopus* transcription factor IIIA has been reported to contain repeated minidomains with zinc-binding property [13], much effort has been made to characterize the rapidly expanding class of zinc finger proteins (reviewed in [8]). It became clear that independent or adjacent zinc fingers can be combined as modules which are able to grip specific DNA sequences, and that they are present in many transcription factors. Additional research demonstrated that protein dimerization often facilitates cooperative high-affinity interactions with DNA, resulting in superactivation of gene induction. Such protein dimerization can result in the formation either of homodimers or of heterodimers. A

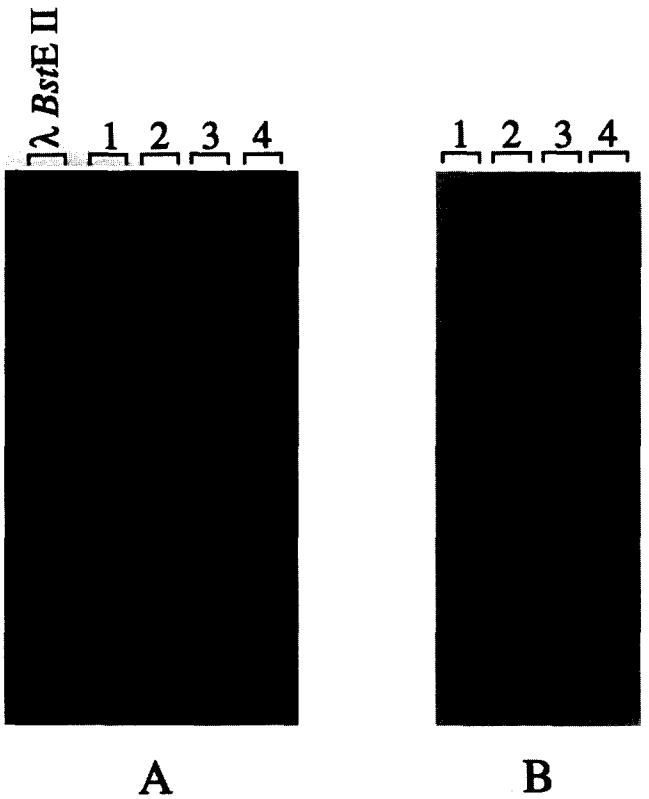

Fig. 3. Detection of A20ZF$^+$ cDNA by Southern blotting. (A) Agarose gel electrophoresis of cDNA inserts (lanes 1–4) after *Eco*RI digestion of positively scoring pGAD424 vectors from an L929r2 cDNA library. (B) Southern blot analysis of the inserts shown in (A), using a ^{32}P-labeled human A20ZF$^+$ fragment as a probe.

well-established example is the transcription factor Sp1 which uses Glu-rich regions for self-interaction [14], but the DNA-binding zinc finger region for heterotypic interaction with, e.g., GATA-1 [15] or RelA(p65) [16]. Self-association of the transcription factor GATA-1 is, however, also mediated by its DNA-binding zinc finger region [17], and the latter is also involved in the interaction with Sp1 [15] or other members of the GATA family of transcription factors [17]. So it has become clear that zinc finger regions, which were initially thought to be involved in DNA binding, can also mediate protein–protein interactions. Alternatively, the LIM domain, another zinc finger motif found in at least 40 different proteins, may be involved in protein–protein interaction, rather than in DNA binding ([18], and reviewed in [19]).

A20 can be considered the first member of a putative novel class of zinc finger proteins consisting of a domain with seven Cys_2/Cys_2 zinc fingers with no structural homology to other known zinc finger motifs [7]. Although A20 has been extensively characterized as an inhibitor of apoptotic and necrotic cell death, its molecular mechanism of action remains largely unknown. Until now, no evidence has been obtained to demonstrate any A20-mediated specific DNA binding. Using the yeast-based two-hybrid system to screen for in vivo protein–protein interactions [20], we here demonstrate that A20 is able to self-associate. Moreover, using C-terminal-deleted or N-terminal-deleted Gal4–A20 fusion proteins, we show that A20 self-association is mediated by the zinc finger domain.

Thus A20 joins the expanding subset of self-associating zinc finger proteins. However, besides self-association, heterotypic protein–protein interaction with A20 is likely to occur and is under further study. Since A20 negatively regulates its own expression, which is mediated by κB elements [2,21], a possible candidate may be the nuclear transcription factor NF-κB.

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References

[1] Dixit, V.M., Green, S., Sarma, V., Holzman, L.B., Wolf, F.W., O'Rourke, K., Ward, P.A., Prochownik, E.V. and Marks, R.M. (1990) J. Biol. Chem. 265, 2973–2978.
[2] Laherty, C.D., Ming Hu, H., Opipari, A.W., Wang, F. and Dixit, V.M. (1992) J. Biol. Chem. 267, 24157–24160.
[3] Sarma, V., Lin, Z., Clark, L., Rust, B.M., Tewari, M., Noelle, R.J. and Dixit, V.M. (1995) J. Biol. Chem. 270, 12343–12346.
[4] Laherty, C.D., Perkins, N.D. and Dixit, V.M. (1993) J. Biol. Chem. 268, 5032–5039.
[5] Opipari, A.W., Ming Hu, H., Yabkowitz, R. and Dixit, V.M. (1992) J. Biol. Chem. 267, 12424–12427.
[6] Sporn, S.A., Eierman, D.F., Johnson, C.E., Morris, J., Martin, G., Ladner, M. and Haskill, S. (1990) J. Immunol. 144, 4434–4441.
[7] Opipari, A.W., Boguski, M.S. and Dixit, V.M. (1990) J. Biol. Chem. 265, 14705–14708.
[8] Klug, A. and Schwabe, J.W.R. (1995) FASEB J. 9, 597–604.
[9] Mertens, N., Remaut, E. and Fiers, W. (1995) Gene 164, 9–15.
[10] Vanhaesebroeck, B., Van Bladel, S., Lenaerts, A., Suffys, P., Beyaert, R., Lucas, R., Van Roy, F. and Fiers, W. (1991) Cancer Res. 51, 2469–2477.
[11] Gietz, R.D. and Schiestl, R.H. (1991) Yeast 7, 253–263.
[12] Tewari, M., Wolf, F.W., Seldin, M.F., O'Shea, K.S., Dixit, V.M. and Turka, L.A. (1995) J. Immunol. 154, 1699–1706.
[13] Miller, J., McLachlan, A.D. and Klug, A. (1985) EMBO J. 4, 1609–1614.
[14] Su, W., Jackson, S., Tjian, R. and Echols, H. (1991) Genes Dev. 5, 820–826.
[15] Merika, M. and Orkin, S.H. (1995) Mol. Cell. Biol. 15, 2437–2447.
[16] Perkins, N.D., Agranoff, A.B., Pascal, E. and Nabel, G.J. (1994) Mol. Cell. Biol. 14, 6570–6583.
[17] Crossley, M., Merika, M. and Orkin, S.H. (1995) Mol. Cell. Biol. 15, 2448–2456.
[18] Feuerstein, R., Wang, X., Song, D., Cooke, N.E. and Liebhaber, S.A. (1994) Proc. Natl. Acad. Sci. USA 91, 10655–10659.
[19] Sanchez-Garcia, I. and Rabbitts, T.H. (1994) Trends Genet. 10, 315–320.
[20] Fields, S. and Song, O. (1989) Nature 340, 245–246.
[21] Krikos, A., Laherty, C.D. and Dixit, V.M. (1992) J. Biol. Chem. 267, 17971–17976.