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Human insulin gene enhancer-binding proteins in pancreatic α and β cell lines

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Electrophoretic mobility shift assays were performed using oligonucleotides corresponding to known protein binding sites within the human insulin gene enhancer and nuclear extracts from mouse pancreatic α and β cell lines. The results demonstrate that a previously described factor, IUF-1, binds to three sites at −82 (the CT1 box), −215 (the CT2 box), and −319 (the CT3 box) in the human insulin gene enhancer. IUF-1 was present only in β but not in α cells, while all other DNA-binding proteins were present in both cell lines. IUF-1 may therefore be an important determinant of insulin gene β cell-specific expression.

Transcription; Gene expression; IUF-1; Islets of Langerhaus

1. INTRODUCTION

The insulin gene is expressed exclusively in the β cells of the islets of Langerhans in adults. The *cis*-acting sequences involved in conferring tissue-specific expression of the insulin gene are located within a region up to 350 base pairs from the transcription start site. Within this region two classes of *cis*-acting element have been shown to play important roles in controlling expression of the gene, E boxes and TAAT boxes [1]. In general E boxes, i.e. the consensus sequence CANNTG, bind protein factors that contain helix-loop-helix (HLH) dimerization domains [2]. In the rat insulin I gene an important regulatory factor, IEF-1 [3], a heterodimer of the E2A gene product (E12/E47) [4–6] and a 25 kDa protein, IESF-1 [7], has been shown to bind to two transcriptionally important E boxes at −103 and −233, the IEB1 or NIR and IEB2 or FAR boxes, respectively [8,9]. IEF-1 is present in islet α and β cells as well as a number of other endocrine cells, but is absent from non-endocrine cells [10,11]. A promoter-proximal TAAT box is located at −86 [10], while three TAAT boxes are located within an AT-rich region (The FLAT element) adjacent to the IEB2/FAR box [12]. The promoter-proximal TAAT box binds a β cell factor, IPF-1 [10], while the FLAT element binds several factors including the homeodomain factors lmx-1, cdx-3, isl-1 and HNF1α [13–15], and an uncharacterised factor called IEF-2 [10]. The FAR and FLAT elements act synergistically as a mini-enhancer [13].

In the human insulin gene the promoter-proximal E box (IEB1/NIR) is conserved, and binds a factor with similar properties to IEF-1 [16]. The distal E box (IEB2/FAR) is not conserved in the human insulin gene, however this region is transcriptionally important [17] and has been identified as a binding site for the ubiquitous HLH protein USF [16]. The human insulin gene contains TAAT box elements at −82 (the CT-1 box), −215 (the CT-2 box) and at −319 (the CT-3 box). The CT-2 box binds a factor called IUF-1 [18]. The human insulin gene also contains a powerful negative regulatory element (NRE) at −270, and several other protein binding sites including the GG1/GG2 motifs at −145 to −130, a cAMP response element (CRE) at −183 to −176, an SP1 site at −346 to −341, and an enhancer core sequence at −314 to −306 [17].

The mouse pancreatic cell lines, αTC1 and βTC3, were derived from transgenic mice expressing the SV40 large T antigen under the control of either the glucagon or insulin enhancer/promoter, respectively [19,20]. Tissue-specific expression of the transgene led to the formation of either glucagonomas or insulinomas, from which immortalised cell lines were established. αTC1 and βTC3 cells produce greater quantities of glucagon or of insulin than other islet cell lines. In addition, they may be closer to terminally differentiated cells than other islet cell lines, such as HIT T15 and RIN m5F

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Abbreviations: CAT, chloramphenicol acetyl transferase; HLH, helix loop helix; NRE, negative regulatory element.

cells which produce comparatively low amounts of insulin, and are heterogeneous in phenotype, to some extent expressing the genes for glucagon and somatostatin as well as the insulin gene [21,22]. For this reason αTC1 and βTC3 cells provide an interesting system for the study of tissue-specific islet hormone gene expression. The aim of the present study was to investigate the presence of human insulin gene enhancer-binding proteins in nuclear extracts from these two cell lines.

2. EXPERIMENTAL

2.1. *Cell lines*

Mouse pancreatic αTC1 and βTC3 cell lines were obtained from S. Efrat, Department of Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY.

2.2. *Cell transfections and chloramphenicol acetyl transferase (CAT) assays*

The plasmid constructs pBCins-339 and pBCins-258 contain fragments of the human insulin gene from −339 to +112 or −258 to +112 cloned into the expression vector pBCO [23]. Cells were transfected with 15 μg of reporter DNA and 6 μg of RSV-β galactosidase DNA by the calcium phosphate co-precipitation method [24]. CAT assays were performed after normalization to β galactosidase activity as described by Gorman et al. [25].

2.3. *Oligonucleotides*

Oligonucleotides were purchased from Alta Bioscience, University of Birmingham. Single stranded complementary oligonucleotides were annealed as previously described [26]. The following oligonucleotides corresponding to the indicated sequences in the human insulin gene were used: oligonucleotide J, which contains the human IEB1 site (underlined) (−122 to −93, 5′-CTCAGCCCCCAGCCATCTGCC-GACCCCCCC-3′); oligonucleotide D, which contains the human IEB2 site (−248 to −219, 5′-ACAGGTCTGGCCACCGGGCCCC-TGGTTAAG-3′); oligonucleotide P, which contains the CT1 box (−92 to −68, 5′-CCCAGGCCCTAATGGGCCAGG-3′); oligonucleotide B, which contains the CT2 box (−230 to −201, 5′-CCCCTG-GTTAAGACTCTAATGACCCGCTGG-3′); oligonucleotide Bm2, which contains an A to C transversion within the CT2 box of oligonucleotide B (−230 to −201, 5′- CCCCTGGTTAAGACTCTACTG-ACCCGCTGG-3′); and oligonucleotide H, which contains the CT3 box (−326 to −290, 5′-TCCTGGTCTAATGTGGAAAGTGGCCC-AGGTGAGGGC-3′). Oligonucleotide P1 contained sequences −85 to −64 of the rat insulin I gene (5′-GCCCTTAATGGGCCAAACG-GCA-3′), while oligonucleotide P1m2 contained the change AA to CC (underlined) within oligonucleotide P1 (5′-GCCCTTCCTGGGCCA-AACGGCA-3′).

2.4. *Electrophoretic mobility shift assays*

Preparation of nuclear extracts and electrophoretic mobility shift assays were performed as previously described [26,27].

3. RESULTS AND DISCUSSION

αTC1 and βTC3 cells were transfected with the constructs pBCins-339 and pBCins-258 containing fragments from the region upstream of the human insulin gene, and with pRSVCAT as a positive control. Although the positive control gave high activity in both cell lines, the insulin promoter constructs were approximately ten-fold more active in βTC3 than in αTC1 cells when normalised against pRSVCAT (Table I). Construct pBCins-258 was approximately 3-fold more active than construct pBCins-339 in both cell lines, indicating that a negative regulatory element (NRE) located between −258 and −279 was active in α and β cells. These results demonstrate that αTC1 and βTC3 cells differ substantially in their ability to support insulin gene transcription. The following experiments were performed to determine to what extent this difference would be reflected in the pattern of expression of insulin enhancer-binding proteins between the two cell lines.

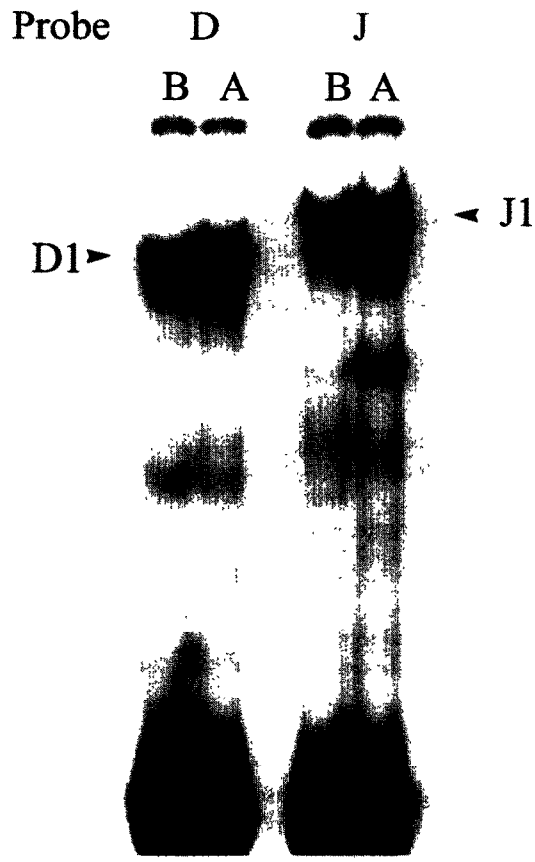

Fig. 1. Binding of αTC1 and βTC3 nuclear proteins to oligonucleotides D and J. Electrophoretic mobility shift assay using oligonucleotides D or J as probes and αTC1 (A) and βTC3 (B) nuclear extracts. The position of the major complexes, J1 and D1, are indicated.

The distribution of factors binding to the proximal E box (IEB1/NIR) and the imperfect distal E box at −233

Table I

Transfection of βTC3 and αTC1 cells with pRSVCAT, pBCins-258 and pBCins-339

Cell line	pRSVCAT	pBCins-258	pBCins-339
βTC3	100	14.6 ± 6.4 (4)	4.4 ± 0.4 (4)
αTC1	100	1.4 ± 0.6 (4)	0.54 ± 0.1 (4)

For each cell line, recovered CAT activities were expressed relative to pRSVCAT. The values are the means of four independent determinations.

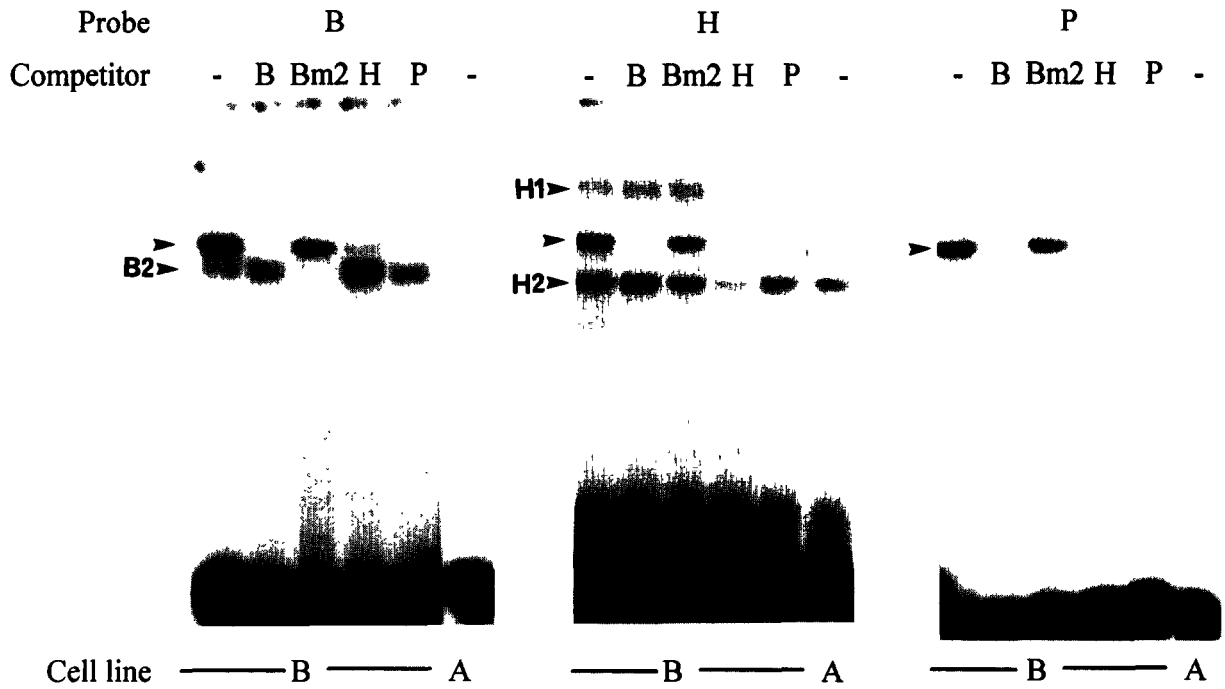

Fig. 2. Binding of αTC1 and βTC3 nuclear proteins to oligonucleotides B, H and P. Electrophoretic mobility shift assay using oligonucleotides B, H or P as probes and αTC1 (A) and βTC3 (B) nuclear extracts. The indicated unlabelled competitor oligonucleotides were used in approximately 50-fold excess. The position of the IUF-1 complex is indicated.

was investigated using oligonucleotides J and D (Fig. 1). A factor, previously identified [16] as the HLH heterodimer IEF-1 (Fig. 1, J1), was detected in both αTC1 and βTC3 cells using oligonucleotide J as probe. Using oligonucleotide D as a probe, a factor, previously identified [16] as the HLH protein USF (Fig. 1, D1) was also present in both cell lines.

To investigate the binding specificity and distribution of factors binding to the three TAAT box sequences in the human insulin gene, three oligonucleotides (H, −325 to −190; B, −230 to −201; and P,−92 to −68) were used. A single prominent complex with identical electrophoretic mobility (Fig. 2) was observed using all three oligonucleotide probes. Self-competition and cross-competition for formation of this complex was observed with oligonucleotides B, H and P, but not with oligonucleotide Bm2, which contains an A- to C- transversion within the TAAT box element of oligonucleotide B. These results are consistent with the conclusion that the factor IUF-1 binds to the CT element [T/C]CTAATG within oligonucleotides B, H, and P. IUF-1 was present in the βTC3 extract, but was absent from the αTC1 extract (Fig. 2, tracks A). A faster mobility complex (B2) was observed with oligonucleotide B, however this complex was not abolished by excess unlabelled oligonucleotide. Unexpectedly, oligonucleotide Bm2 did compete for formation of complex B2. It is likely that complex B2 represents non-specific protein interaction with the probe. Oligonucleotide H also produced two additional complexes H1 and H2. Both represented specific protein–DNA interactions since self-competition with oligonucleotide H was observed. Complex H1 was present in βTC3 but not αTC1 extracts. It exhibited competition with oligonucleotide P but not with B or Bm2. Complex H2 was present in both βTC3 and αTC1 extracts. Both complexes require further characterization. In theory either complex might represent binding of proteins at the enhancer core sequence within oligonucleotide H.

The binding of αTC1 and βTC3 nuclear proteins to oligonucleotide B was further investigated over a range of salt concentrations. In this study two complexes were observed using the βTC3 extract (Fig. 3, arrows). The upper complex has a mobility similar to that of IUF-1. The lower complex has been described previously [28], and represents proteolytic breakdown of IUF-1. The IUF-1 complex was unaffected by salt over the range 50–150 mM. In fact IUF-1 will bind to its TAAT box sequence at salt concentrations up to 500 mM (data not shown). Using the αTC1 extract no significant binding activity was observed at 50 mM salt. At 100 mM and 150 mM salt two low mobility complexes appeared. Again these have been described previously and shown to represent binding of proteins at sites within oligonucleotide B that do not include the IUF-1 binding sequence TCTAATG [28]. These results lend further support to the conclusion that IUF-1 is expressed exclusively in the βTC3 cells but not in the αTC1 cells.

Ohlsson et al. [10] have reported that a factor, IPF-1, that is present in βTC cells but absent from αTC cells

bound to an oligonucleotide (P1) containing sequences from −85 to −64 of the rat insulin I gene [10]. This sequence contains a TAAT box, and mutagenesis of the TAAT sequence to TCCT abolished the binding of IPF-1. To determine the relationship between IPF-1 and IUF-1, competition experiments were performed using the human insulin gene probe P and the rat insulin I gene P1 and P1m2 oligonucleotides as competitors. The result showed that oligonucleotides P and P1 competed for formation of the IUF-1 complex, while oligonucleotide P1m2 failed to compete. Thus IUF-1 and IPF-1 may be similar or closely related factors.

The binding of αTC1 and βTC3 nuclear proteins to oligonucleotide probes corresponding to the GG1 and GG2 sites, the CRE, the NRE [17], and the SP1 site at −346 to −341 was also studied. However, no substantial difference between the cell extracts was observed with any of these probes (data not shown).

In conclusion, we have confirmed that the HLH heterodimer, IEF-1, that binds to the IEB1/NIR and IEB2/FAR sequences in the rat insulin I gene and the IEB1/NIR sequence in the human insulin gene is present in both αTC1 and βTC3 cells. The HLH factor USF that binds to the IEB2/FAR-equivalent sequence of the human insulin gene is also present in both cell types. This is in keeping with the widespread tissue distribution of this factor [29]. The factor IUF-1 was shown to bind to three sites in the human insulin gene, the CT1, CT2 and CT3 boxes, and to be present in βTC3 cells but absent from αTC1 cells. IUF-1 and the previously described factor IPF-1 [10], are restricted to β cells and exhibit a similar binding specificity. They may represent similar factors, that exhibit the most tightly restricted tissue specificity of any insulin gene enhancer binding protein so far described. Interestingly, IUF-1 has also been shown to bind to an important regulatory sequence in the β cell-specific glucokinase promoter [30], indicating that it may play an important role in β cell specific gene expression. Further investigation of this hypothesis and the potential role of IUF-1 in the glucose-mediated stimulation of insulin gene transcription [31] must await cloning of the cDNA for IUF-1.

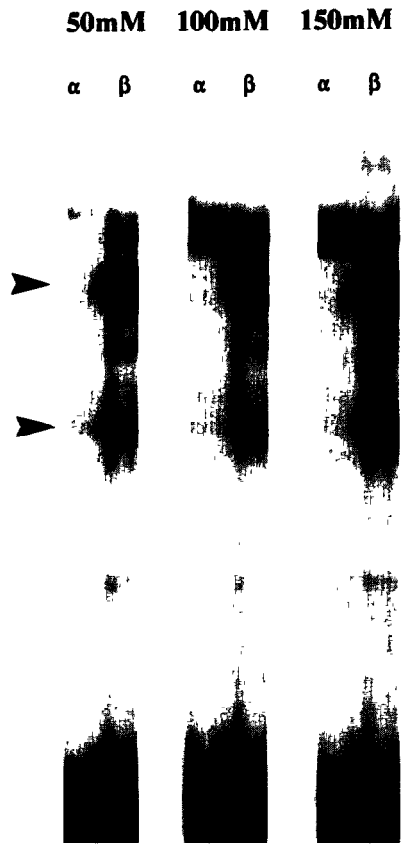

Fig. 3. Effect of KCl on the binding of αTC1 and βTC3 nuclear proteins to oligonucleotide B. Electrophoretic mobility shift assay using oligonucleotides B as probe and αTC1 and βTC3 nuclear extracts. Binding reactions were performed with the indicated final KCl concentration. The upper arrow points to the IUF-1 complex, while the lower arrow points to a complex form as result of proteolysis of IUF-1.

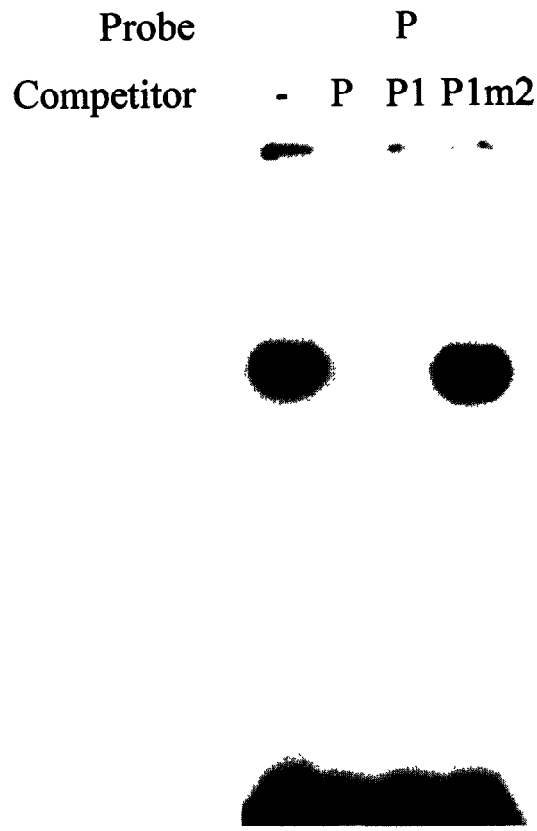

Fig. 4. Binding of βTC3 nuclear proteins to oligonucleotide P. Electrophoretic mobility shift assay using oligonucleotide P as probe and a βTC3 nuclear extracts. The indicated unlabelled competitor oligonucleotides were used in approximately 50-fold excess.

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REFERENCES

[1] Clark, A.R. and Docherty, K. (1993) Biochem. Soc. Trans. 21, 154–159.
[2] Olson, E.N. (1990) Genes and Development 4, 1454–1461.
[3] Karlsson, O., Edlund, T., Moss, L.B., Rutter, W.J. and Walker, M.D. (1987) Proc. Natl. Acad. Sci. USA 84, 8819–8823.
[4] Walker, M.D., Park, C.W., Rosen, A. and Aronheim, A. (1990) Nucleic Acids Res. 18, 1159–1166.
[5] Shibasaki, Y., Sakura, H., Takaku, F. and Kasuga, M. (1990) Biochem. Biophys. Res. Commun. 170, 314–321.
[6] Nelson, C., Shen, L.-P., Meister, A., Fodor, E. and Rutter, W.J. (1990) Genes and Development 4, 1035–1043.
[7] Park, C.W. and Walker, M.D. (1992) J. Biol. Chem. 267, 15642–15649.
[8] Karlsson, O., Walker, M.D., Rutter, W.J. and Edlund, T. (1989) Mol. Cell. Biol. 9, 823–827.
[9] Moss, L.G., Moss, J.B. and Rutter, W.J. (1988) Mol. Cell. Biol. 8, 2620–2627.
[10] Ohlsson, H., Thor, S. and Edlund, T. (1991) Mol. Endocrinol. 5, 897–903.
[11] Aronheim, A., Ohlsson, H., Park, C.W., Edlund, T. and Walker, M.D. (1991) Nucleic Acids Res. 19, 3893–3899.
[12] German, M.S., Moss, L.G., Wang, J. and Rutter, W.J. (1992) Mol. Cell. Biol. 12, 1777–1788.
[13] German, M.S., Wang, J., Chadwick, R.B. and Rutter, W.J. (1992) Genes and Development 6, 2165–2176.
[14] Karlsson, O., Thor, S., Norberg, T., Ohlsson, H. and Edlund, T. (1990) Nature 344, 879–882.
[15] Emens, L.A., Landers, D.W. and Moss, L.G. (1992) Proc. Natl. Acad. Sci. USA 89, 7300–7304.
[16] Read, M.L., Clark, A.R. and Docherty, K. (1993) Biochem. J. (in press).
[17] Boam, D.S.W., Clark, A.R. and Docherty, K. (1990) J. Biol. Chem. 265, 8285–8296.
[18] Boam, D.S.W. and Docherty, K. (1989) Biochem. J. 264, 233–239.
[19] Efrat, S., Teitelman, G., Anwar, M., Ruggerio, D. and Hanahan, D. (1988) Neuron 1, 605–613.
[20] Efrat, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D. and Baekkeskov, S. (1988) Proc. Natl. Acad. Sci. USA 85, 9037–9041.
[21] Madsen, O.D., Larsson, L.-I., Rehfeld, J.F., Schwartz, T.W., Lernmark, Å., Labrecque, A.D. and Steiner, D.F. (1986) J. Cell Biol. 103, 2025–2034.
[22] Philippe, J., Chick, W.L. and Habener, J.L. (1987) J. Clin. Invest. 79, 351–358.
[23] Clark, A.R., Boam, D.S.W. and Docherty, K. (1989) Nucleic Acids Res. 17, 1013.
[24] Docherty, K. and Clark, A.R. (1993) in: Gene Transcription: A Practical Approach (Hames, B.D. and Higgins, S.J. Eds.) pp. 64–123, Oxford University Press, Oxford.
[25] Gorman, C., Moffat, L. and Howard, B. (1982) Mol. Cell. Biol. 2, 1044–1051.
[26] Boam, D.S.W., Clark, A.R., Shennan, K.I.J. and Docherty, K. (1990) in: Peptide Hormone Secretion: A Practical Approach (Hutton, J.C. and Siddle, K. Eds.) pp. 271–307, Oxford University Press, Oxford.
[27] Schreiber, E., Matthias, P., Müller, M.M. and Schaffner, W. (1989) Nucleic Acids Res. 17, 6419.
[28] Scott, V., Clark, A.R., Hutton, J.C. and Docherty, K. (1991) FEBS Lett. 290, 27–30.
[29] Sawadogo, M. and Roeder, R.G. (1985) Cell 43, 165–175.
[30] Shelton, K.D., Franklin, A.J., Khoor, A., Beechem, J. and Magnuson, M.A. (1992) Mol. Cell. Biol. 12, 4578–4589.
[31] Melloul, D., Ben-Neriah, Y. and Cerasi, E. (1993) Proc. Natl. Acad. Sci. USA 90, 3865–3869.