Vol. 204, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 14, 1994 Pages 366-374

Recognition DNA sequence of a novel putative transcription factor, BCL6

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Received September 6, 1994

The BCL6 gene involved in the 3q27 translocation associated with B-cell lymphomas encodes a novel Cys2-His2 zinc finger protein. We generated a fusion protein of glutathione S-transferase and zinc finger domain of BCL6 to determine recognition sequences of BCL6 with polymerase chain reaction using random oligonucleotides of 26 bases as a ligand. A consensus of 14 nucleotides consisting of (T/A)NCTTTCNAGG(A/G)AT was identified in the recognition sequences. In a gel mobility shift assay, the probe containing the 14-nucleotide recognition sequence formed a complex with the fusion protein and nuclear proteins from Burkitt's cell lines overexpressing the *BCL6* transcripts. The consensus sequence was protected from the digestion by nuclease in a DNase I footprinting assay. In conclusion, BCL6 may be involved in tumorigenesis by binding to the consensus sequences of the other genes. © 1994 Academic Press, Inc.

The translocation involving band 3q27 is one of non-random chromosomal abnormalities in B-cell malignancies, especially diffuse large cell lymphoma [1,2]. We have previously cloned a putative proto-oncogene, *BCL6*, on band 3q27, which is also designated as LAZ3 or *BCL5* (we have previously reported it as *BCL5*) [3-5]. The rearrangements of the *BCL6* gene have been found in patients with malignant lymphoma [6-8]. As a result of the rearrangement, the regulatory region of the *BCL6* gene is replaced by other genes including those of the immunoglobulin [3,5,9,10], leading to overexpression of the

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BCL6 gene. In several Burkitt's cell lines, including Raji, Daudi and Ramos, the *BCL6* gene is also overexpressed [4], although they do not have any rearrangement involving the *BCL6* gene.

The *BCL6* gene encodes a 79-kDa protein and the deduced amino acid sequence has a Krüppel type zinc finger motif, or six Cys2-His2 zinc fingers, which is one of DNA binding motifs [11,12]. In this study, we determined recognition sequences of BCL6 with polymerase chain reaction (PCR) using random oligonucleotides of 26 bases as a ligand [13].

Methods and Materials

Construction and production of GST-*BCL6* fusion protein. A *ScaI-XhoI* fragment of the *BCL6* cDNA encoding the zinc finger domain (B6ZF) was fused in frame downstream to glutathione S-transferase (GST) in pGEX-4T-3 vector (Pharmacia, Uppsala, Sweden) (Figs. 1A). Following one-hour incubation of a 10 ml culture with 0.1 mM isopropyl-β-thiogalactopyranoside, the bacterias transformed by the construct were collected by centrifugation, washed twice in cold phosphate-buffered saline (PBS), resuspended in 0.5 ml of PBS, and lysed by sonication. The fusion protein was isolated from the supernatants with 500 μl of a slurry of 50% glutathione-Sepharose beads (Pharmacia). GST protein without B6ZF was generated from pGEX-4T-3 vector. The protein sizes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Oligonucleotides. The 64-base random oligonucleotides of N26 (5' GGCTGAGTCTGAACGGATCCN_{26}CCTCGAGACTGAGCGTCG 3') as a template for second strand, primer 1 (5' CGACGCTCAGTCTCGAGG 3') and primer 2 (5' GGCTGAGTCTGAACGGATCC 3') were synthesized [14].

Selection of GST-B6ZF fusion protein-binding oligonucleotides from random oligonucleotides. Binding selection was performed according to the method reported by Delwel et al. [13]. Ten μl aliquot containing 200 pmol double-stranded DNA of oligonucleotides N26 was mixed with 50 μl of a 50% slurry of GST-B6ZF fusion protein bound to glutathione-Sepharose beads. After 30-min incubation at room temperature, the selected oligonucleotides were eluted from the beads in 50 μl of H_2O by boiling for 5 min. The supernatant was used for PCR amplification with primers 1 and 2. The amplified oligonucleotides were subjected to 5 additional cycles of binding reaction and amplification. The final PCR product was sequenced by a M13 reverse primer on Autosequencer 373A (Applied Biosystem, Foster City, CA).

Gel mobility shift assay with oligonucleotides. Three kinds of double-stranded oligonucleotides with different nucleotides at positions 1 and 12 were used. They were TA probe (5'-TGCT TTCTAGGAAT-3'), AA probe (5'-ATCTTTCTAGGAAT-3') and TG probe (5'-TGCTTTCTAGGGAT-3') with same flanking sequences at both sides.

The ^{32}P-radiolabeled double-stranded oligonucleotide ($2x10^4$ cpm; approximately 1 to 10 ng) were mixed with the GST-B6ZF fusion protein, GST protein or uclear proteins which were extracted according to the method previously described by Schreiber et al. [15]. Following a 15-min incubation on ice and a 15-min incubation at room temperature, the bound complexes were separated from the free probe on a nondenaturing polyacrylamide gel at 4°C. The gel was vacuum-dried, exposed to an imaging plate and analyzed on an Imaging Analyzing Computer BAS 2000 (Fuji film, Tokyo, Japan).

DNase I footprinting assay. Radiolabeled 64-bp PCR products were subject to DNase I footprinting assay as previously described by Perkins et al. [16].

Results

The fusion protein produced had an expected size of 46kDa as shown in Fig. 1B. The Sepharose-bound protein was used for binding of oligonucleotides. The bound oligonucleotides containing a core of 26 random nucleotides were then amplified and the amplified

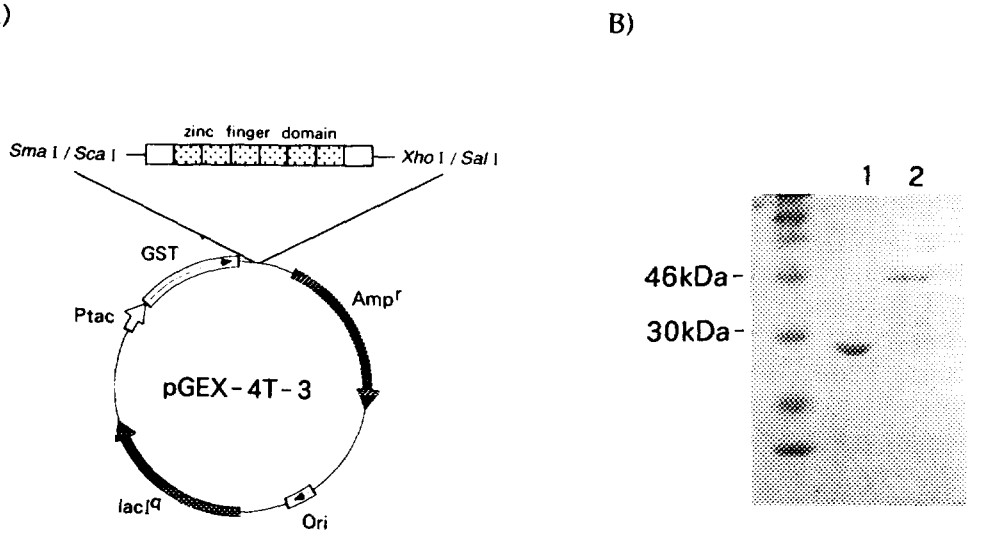

Fig. 1. Production of GST-B6ZF fusion construct. (A) Construction of the GST-B6ZF expression vector. A *Sca* I- *Xho* I fragment of BCL6 cDNA encoding the zinc finger domain was ligated into the *Sma* I- *Sal* I site of pGEX-4T-3 vector to produce GST-B6ZF fusion protein. Amp^r, ampicillin resistance gene; Ori, replication origin; lacI^q, lac repressor with mutation; Ptac, tryptophan-lactose hybrid promoter; GST, glutathione S transferase. (B) SDS-PAGE analysis of the GST-B6ZF fusion protein. The constructs with or without zinc finger motif were introduced into *E. coli* and proteins produced were analyzed in a 12% SDS-PAGE and stained with Coomassie blue. Left column, a Rainbow Marker (Gibco and BRL, Tokyo, Japan); lane 1, a GST protein; lane 2, a GST-B6ZF fused protein.

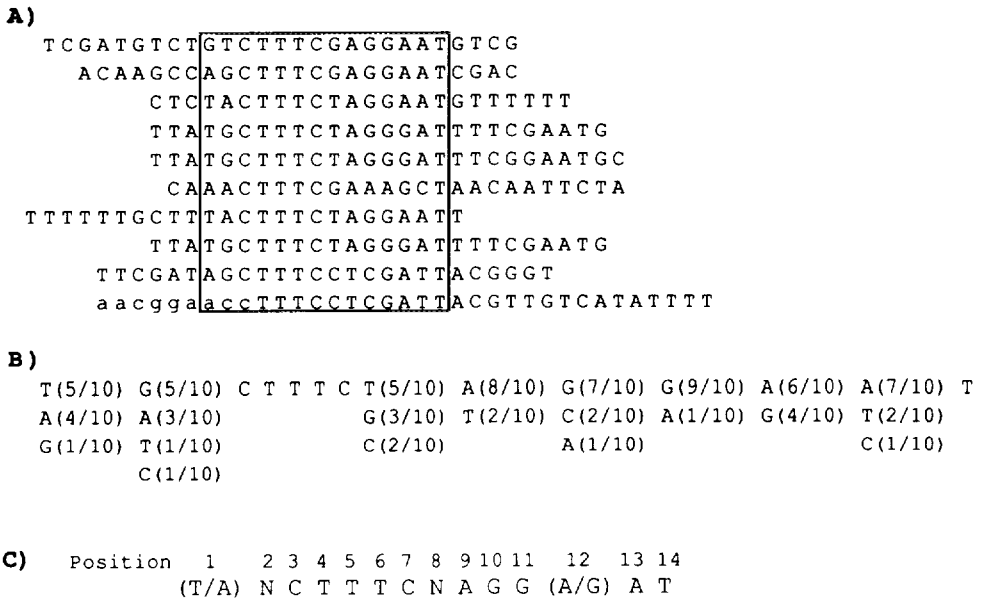

Fig. 2. Consensus sequence of BCL6 DNA recognition sequences. (A) The sequences of the selected and isolated 10 independent clones. The consensus sequences are surrounded by box. Small letters indicate a sequence of a part of a primer. (B) The frequencies of nucleotides used at each position. The frequencies are indicated in parentheses. (C) The 14-bp recognition consensus sequence of BCL6. N means that all four nucleotides are changeable.

oligonucleotides were then subjected to 5 additional cycles of binding and amplification. The resultant oligonucleotides were then cloned, and 10 independent isolates were identified. The sequences of these isolates are shown in Fig. 2A. The consensus sequence of the clones consists of 14-nucleotide sequence (T/A)NCTTTCNAGG(A/G)AT as shown in Fig. 2B. Five nucleotides CTTTC at positions 3 to 7, and two nucleotides at positions 11 and 14 in the consensus sequence were invariant (Fig. 2C). Position 9 had two variations, while positions 10 and 13 had three variations.

To confirm binding-specificity of the protein, we first compared the GST-B6ZF fusion protein with GST protein by a gel mobility shift assay using a synthetic double-stranded TA probe containing the consensus sequence of TGCTTTCTAGGAAT. The GST-B6ZF protein formed a complex with the probe, whereas GST protein alone did not alter the mobility (Fig. 3A). Furthermore, the fusion protein pretreated with a chelating agent of 100 mM EDTA did not alter the mobility of the probe. Addition of excess zinc ions to the reaction mixture containing the fusion protein with 100 mM EDTA restored the activity and altered the

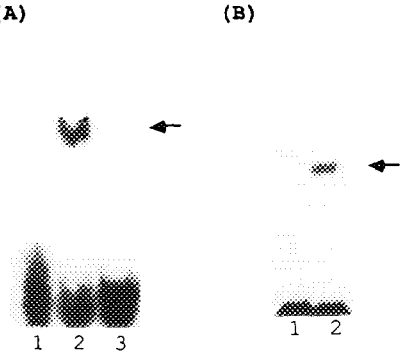

Fig. 3. Gel mobility shift assay using constructed proteins. (A) GST protein (lane 1) and GST-B6ZF fusion protein (lane 2) were mixed with a labeled TA probe as described in Materials and Methods. The shifted band is indicated by an arrow. Lane 3; probe only. (B) GST-B6ZF was pretreated with 100 mM EDTA and then mixed with a labeled TA probe in a buffer in an absence (lane 1) or presence (lane 2) of zinc ions.

mobility of the probe (Fig. 3B). These results indicate that the DNA-binding activity of the fusion protein depends on the zinc finger motif of BCL6 and the presence of zinc ions.

At two sites of the consensus sequence, different nucleotides, T or A at position 1 and A or G at position 12, are similarly used (Figs. 2B and 2C). In gel mobility shift assay using the labeled TA probe with T at position 1 and A at position 12, the cold AA probe with A at position 1 and 12 competed well (Fig. 4A). Using labeled AA probe, similar levels of competition were seen with cold TA probe (Fig. 4B). Two nucleotides, A or G at position 12, of the consensus sequence (TA and TG probes) were examined for the binding affinity. Comparable levels of competition were seen with both probes, suggesting similar binding affinity of two nucleotides of A and G at position 12 (Figs. 4 C and 4D). These indicate that nucleotides A and T at position 1, and A and G at position12 contribute similary for the binding affinity of the consensus sequence for the fusion protein.

Further we performed a gel mobility shift assay using nuclear proteins extracted from Burkitt's cell lines (Raji and Ramos), in which the *BCL6* gene is expressed in a high level [17], and also from T-lymphocytic cell line Molt4, in which the transcript of the *BCL6* gene was not detected by Northern blot analysis. The nuclear proteins extracted from Burkitt's cell lines of Raji and Ramos altered the mobility of the probes, whereas the nuclear protein from Molt4 did not

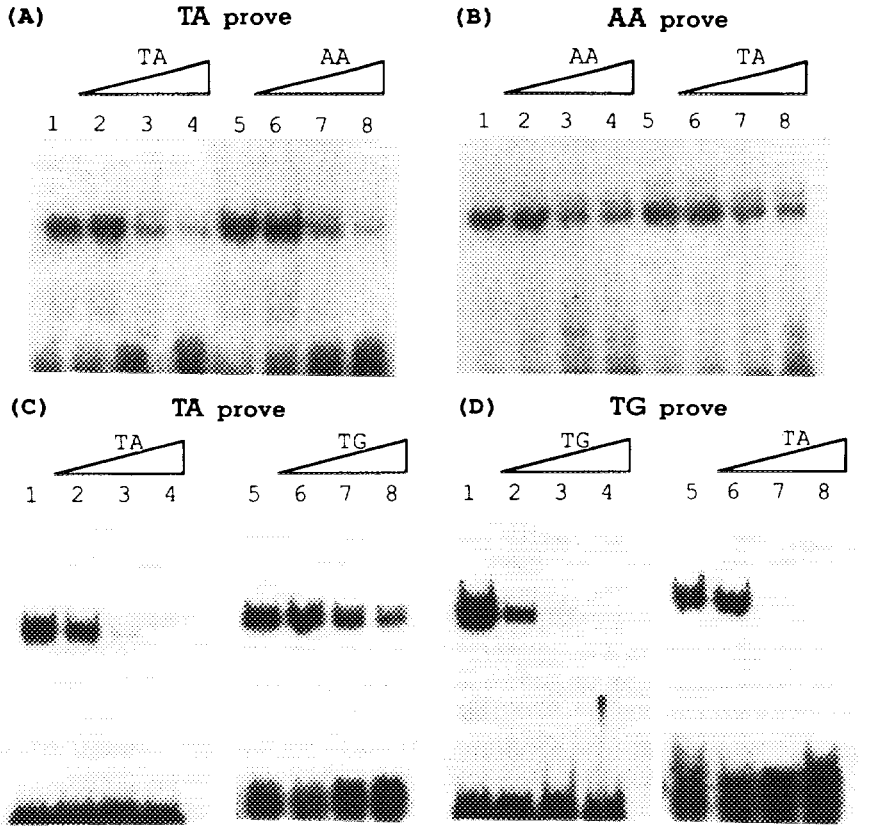

Fig. 4. The gel mobility shift assay using GST-B6ZF protein and competitive probes.
(A) The radiolabeled TA (panels A and C), AA (panel B), or TG (panel D) probes was used for the assay in the absence of cold probes (lanes 1 and 5) or in the presence of increasing amounts of cold probes (lanes 2-4 and 6-8). The amounts of cold probes used were the same as that of the labeled probes in lanes 2 and 6, 10-time excess in lanes 3 and 7, and 50-time excess in lanes 4 and 8. The electrophoresis was carried out on a 8% polyacrylamide gel. Panel A; cold TA or AA probe competed with radiolabeledTA probe, Panel B; Cold TA or TG probe competed with radiolabeled AA probe, Panel C ; Cold TA or TG probe competed with radiolabeled TA probe,Panel D ; Cold TG or TA probe competed with radiolabeled TG probe.

alter the mobility of the probe (Fig. 5). These suggest the specificity of the consensus sequence for the binding to BCL6 expressed in the cells.

We performed DNase I footprinting analysis to confirm the binding of the fusion protein to the consensus sequence of TGTCTTTCGAGGAAT. A 64-bp double-stranded DNA fragment containing the consensus binding site was end-labeled with ^{32}P and used for the analysis. The region of the consensus sequence of TGTCTTTCGAGGAAT

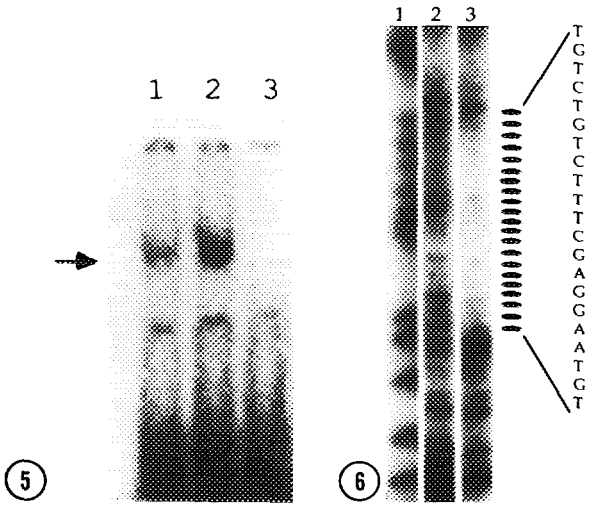

Fig. 5. Gel mobility shift assay using nuclear proteins extracted from the cell lines. The nuclear proteins extracted from Burkitt's cell lines of Raji (lane 1) and Ramos (lane 2), and T-lymphocytic cell line of Molt4 (lane 3) were used. The electrophoresis was carried out on a 4% polyacrylamide gel. The position of shifted bands are indicated by an arrow. Non-specfic bands are detected at a position below the shifted band in all lanes.

Fig. 6. Protection of binding site by GST-B6ZF fusion protein. GST-B6ZF protein was incubated with an end-labeled 64-bp probe containing the consensus sequence and then partially digested with DNase I. Fragments were fractionated on a 8% denaturing polyacrylamide gel. Lane 1 shows the pattern of the thymidine (T). Lanes 2 and 3 show the patterns of digestion in the absence or presence of the protein, respectively. The protected sequence is indicated by marks and letters.

with the extension of 4-bp at 5' and 2-bp at 3' sites was completely protected by the fusion protein of GST-B6ZF from the digestion by nuclease (Fig. 6). This indicates that the fusion protein actually bound to the consensus sequence.

Discussion

In this study, we identified BCL6 binding sequences of (T/A)NCTTTCNAGG(A/G)AT. To identify the BCL6 binding site, we used a technique described previously by Delwel et al. [13]. Two-hundred pmoles of the double-stranded N26 random oligonucleotide used for the binding reaction contain approximately 1.2×10^{13} molecules, which are sufficient to ensure that all possible combinations of the 26-

nucleotide randomized region (4^{26}= $6.9x10^{10}$) would be represented in each binding reaction. Most sequence-specific DNA-binding zinc finger proteins contain fewer than 10 zinc fingers that are usually clustered in a single tandem array [18]. Since each finger usually interacts with 3 to 5 nucleotides [18], 6 zinc fingers of BCL6 are calculated to interact with 18 to 30 nucleotides. In some zinc finger proteins, only a part of all fingers contributes to the DNA-recognition [13]. Therefore 26 random nucleotides used are sufficient for identification of recognition sequences for BCL6.

We next examined the affinity of BCL6 for the consensus sequences which have different nucleotides at positions 1 and 12. The consensus sequences with different nucleotides at positions 1 (A or T) and 12 (T or G) function similarly for DNA-binding affinity. Further we confirmed the bindings of the DNA-binding domain of BCL6 to the consensus sequence by DNase I footprinting analysis.

In Burkitt's cell lines, Raji and Ramos, transcript of the *BCL6* gene is overexpressed [4] and the gel mobility shift assay revealed formation of a complex of the oligonucleotides containing the consensus sequence with nuclear proteins of these cell lines. This suggests that the BCL6 proteins might regulate transcription of another gene(s).

To our knowledge, the DNA sequence (T/A)NCTTTCNAGG(A/G)AT which we have identified as an BCL6 binding site is distinct from the DNA-binding sites for other DNA-binding proteins. The consensus sequences do not appear to be palindromic or tandem repeat. By searching the GenBank and EMBL DNA data bases; the software package of the DNASIS (Hitachi Softwear Engineering, Yokohama, Japan) for the consensus sequence or close variations thereof, we have found that these sequences are present in the 5'-regions of the glucokinase gene [19] and secretory leukoprotease inhibitor protein gene [20], and intronic region of the dystrophin gene [21]. By inteacting with the regions of these genes, BCL6 may play an important role in the tumorigenesis of B-cell malignancies.

Acknowledgment

This study was supported by a Grant-in-Aid for Scientific Research on Priority Areas (06280202) from the Ministry of Education, Science and Culture of Japan to S.H.

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