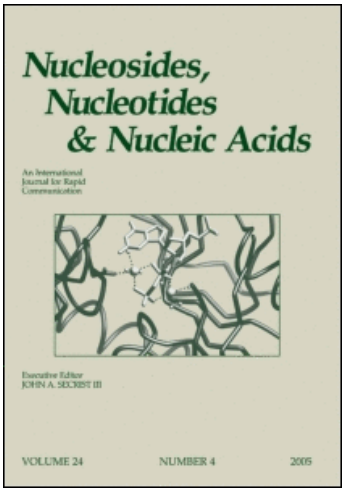

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BCL-2 Regulation Targeting the AU-Rich Domain

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BCL-2 REGULATION TARGETING THE AU-RICH DOMAIN

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ABSTRACT: In an effort to identify potential upregulators of *bcl-2* activity in t(14;18) follicular B lymphoma cells, we detected a hybrid *bcl-2*/IgH RNA transcribed in antisense orientation. This antisense transcript may contribute to upregulation of *bcl-2* expression in t(14;18) cells, overlapping AU-rich motifs present in the 3'-untranslated region of *bcl-2* mRNA. We have studied the enzymatic efficiency of a ribozyme directed towards the *bcl-2* AU-rich region in a cell-free system determining its kinetic parameters.

The prevailing karyotypic abnormalities related to hematological malignancies are chromosomal translocations[1,2] which give rise to hybrid genes either endowed with deregulated expression[3,4] or producing hybrid proteins[5,6]. The t(14;18)(q32;q21) is consistently associated with most follicular and diffuse B-cell lymphomas. When the truncated chromosomes are juxtaposed, a hybrid *bcl-2*/IgH fusion gene originates that does not disrupt the *bcl-2* open reading frame or produce a hybrid protein[7]. Instead, higher levels of *bcl-2*/IgH hybrid mRNA and of normal BCL-2 protein are present in the t(14;18) B-cells than in the t(14;18)-negative counterparts[7-9]. The BCL-2 protein has been shown to prevent programmed cell death, providing a cell survival advantage[8,10-12] that appears to be implicated in neoplastic transformation[13,14].

In recent years we have investigated the response of follicular lymphoma cells to oligonucleotides, including those targeting N-region insertions[15] (stretches of DNA

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inserted during *bcl-2*/IgH recombination) whose sequence is unique to each individual lymphoma[7]. These studies have unexpectedly led us to identify in these lymphomas a hybrid *bcl-2*/IgH transcript in antisense orientation[16], originating in the IgH locus and encompassing the N region and part of the 3' untranslated region (3'UTR) of the *bcl-2* gene. It is thought that it contributes to *bcl-2* overexpression and, therefore, to oncogenicity[17], although direct evidence to support this hypothesis is lacking. We have hypothesized that this antisense transcript might act by functionally inactivating the negative regulatory regions such as the AU-rich RNA-destabilizing element (ARE)[18,19] that we have identified in the 3'-UTR of *bcl-2* mRNA[16].

The *bcl-2* ARE sequence corresponds to a region of 406 nucleotides in the 3'UTR of the human *bcl-2* mRNA. In this region, a 107-nucleotide element is endowed with the features known in the other mRNA destabilizing AREs. We recently demonstrated the effect of *bcl-2* ARE activity on RNA half-life[20]. The ARE region of *bcl-2* mRNA was chosen as the target for ribozyme-catalyzed cleavage in a cell-free system. Ribozymes provide a relatively simple yet effective catalytic sequence capable of enzymatically cleaving a wide range of RNA sequences[21,22].

A synthetic *bcl-2* ARE RNA from position 2179 to 2567 was obtained by in vitro transcription, labeled by incorporation of DIG-UTP and designed as ARE RNA. A control transcript was obtained in the same way from the pTRI-IGFR plasmid and designed as IGFR RNA. RZ3-BL2 ribozyme consisted of the conserved core region and two arms at the 5' and 3' termini which directed the ribozyme to the ARE sequence (Fig. 1). The cleavage took place at position 2372. We incubated the transcripts and the ribozyme at 37°C and started the reaction by adding $MgCl_2$. The reactions were stopped by denaturing the RNA and ribozyme complexes present in the mixture. Digoxigenin-labeled RNA was electrophoresed, transferred to a filter and detected with the digoxigenin system (Fig. 2). RZ3-BL2 cleaved ARE RNA precisely and efficiently under physiological conditions in this cell-free system (lanes A-E). Cleavage was specific: the sizes of cleaved fragments corresponded to those expected from cleavage immediately 3' proximal to nucleotide 2372. As a control for specificity, we used the IGFR transcript. As expected, no cleavage product was present (lane G). The ribozyme activity was dependent on $MgCl_2$ (lane B), in agreement with other reports[23-26]. Using three different concentrations of ribozyme (100 nM, 10 nM, 1nM), we noted that the amount of cleavage product was dose-dependent (Fig. 2B).

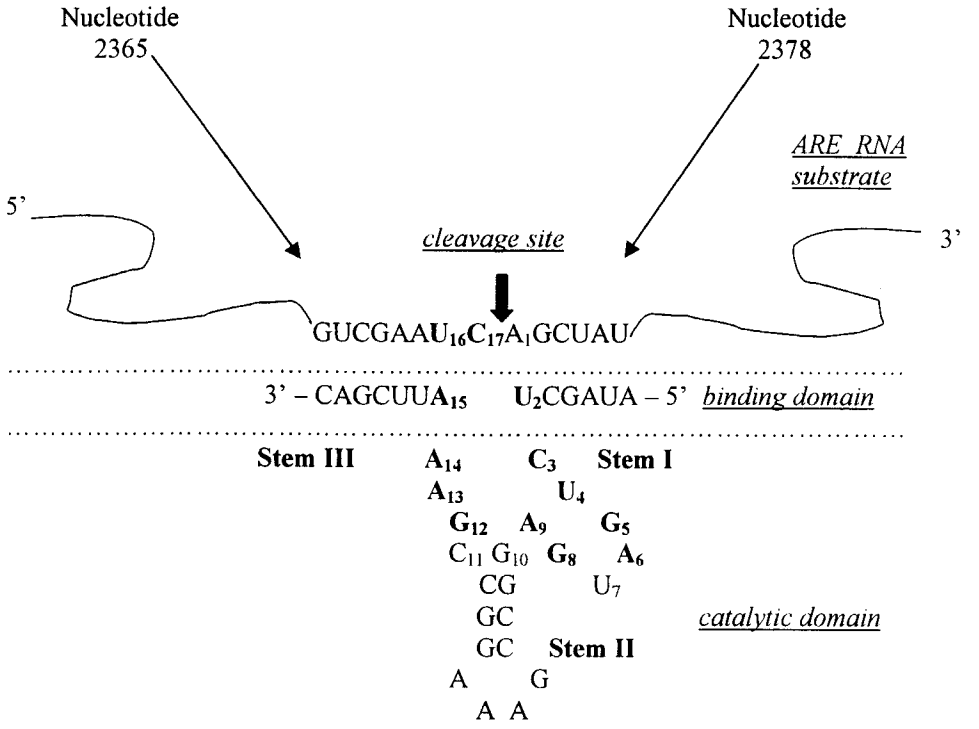

FIG. 1. Structure of hammerhead ribozyme RZ3-BL2 and its target (ARE region of *bcl-2* mRNA). The cleavage site is indicated by an arrow.

The catalytic efficiency of ribozyme RZ3-BL2 was measured in a cell-free system by kinetics experiments. Two concentrations of RZ3-BL2 (10 nM and 3.3 nM) were used at 37°C for 3 hours, and kinetic parameters were determined[23]. The ratio K_{react}/K_m, often used as a measure of the relative efficiency of enzymatic activity, was 0.003 (min^{-1} nM^{-1}) (Table 1). Comparing this parameter with those reported elsewhere[27-30] we conclude that the catalytic efficiency of RZ3-BL2 is good. The catalytic properties of RZ3-BL2 appear very encouraging for its use in cellular systems. Ribozymes interacting with the ARE sequence could be used to modulate *bcl-2* mRNA stability.

MATERIALS AND METHODS

Cell lines. The human follicular B cell lymphoma lines $DOHH_2$[31] and K422[32], carrying the 14;18 chromosomal translocation, and the human t(14;18)-negative Raji B

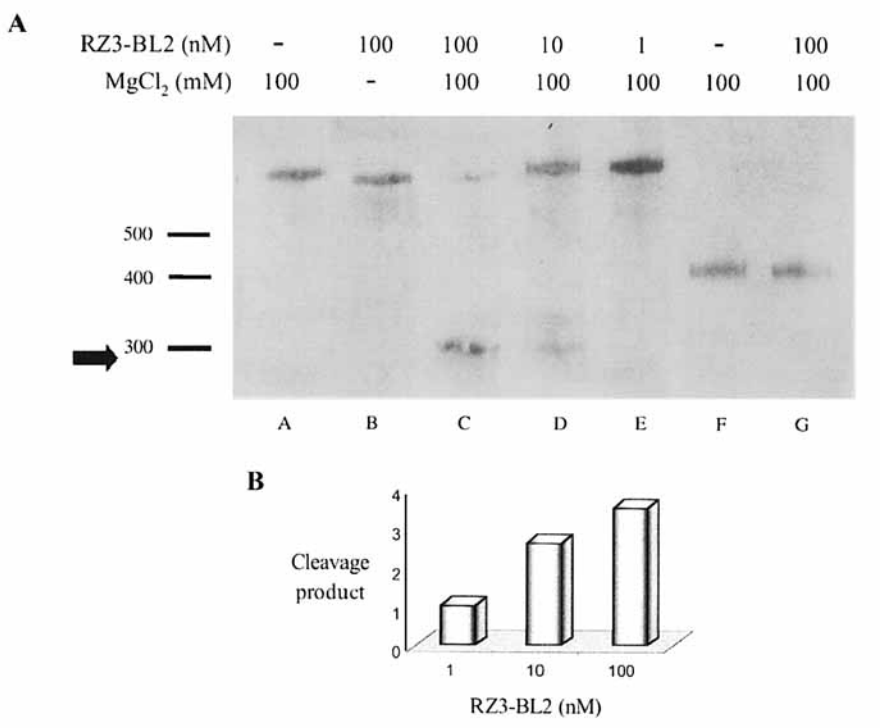

FIG. 2. Ribozyme-mediated cleavage reaction at 37°C.
(A) Lanes A-E: the DIG-labeled RNA substrate is *bcl-2* ARE. The cleavage product is indicated by an arrow. Lanes F-G: the control DIG labeled substrate is IGFR. On the left, RNA length sizes (nucleotides). (B) Dose dependence of cleavage product formation.

TABLE 1. Kinetic constants of RZ3-BL2 ribozyme determined in a cell-free system

RIBOZYME	K_{react} (10^{-3} min^{-1})	K_m (nM)	K_{react}/K_m (min^{-1} nM^{-1})
RZ3-BL2	6	2.05	0.003

(Burkitt lymphoma) cell line[33] were grown in RPMI 1640 medium containing 10% heat inactivated fetal calf serum plus glutamine.

Ribozyme. (RZ3-BL2) hammerhead ribozyme was directed against nucleotides 2365-2378 of *bcl-2* mRNA. Cleavage site was after nucleotide 2372 of *bcl-2* ARE. The ribozyme sequence is 5'-AUAGCUCUGAUGAGGCCGAAAGGCCGAAAUUCGAC-3' (35-mer). It was gently supplied by Lorenzo Citti (CNR-Pisa, Italy).

In vitro transcription. Transcripts were synthesized *in vitro* using Sp6 RNA polymerase according to the procedure described by the supplier (Riboprobe Combination System, Promega, Madison, WI) incorporating DIG-labeled NTPs (Hoffmann-La Roche, Basel, Switzerland). The plasmid pCR-U1[20] was linearized with the restriction enzyme Apa I (New England Biolabs, Beverly, MA). The control plasmid pTRI-IGFR (Ambion, Austin, TX) was linearized with the restriction enzyme Bam HI (Amersham, Uppsala, Sweden).

Ribozyme mediated cleavage. Standard cleavage reactions involved heating to 50°C for 5 minutes of separate tubes containing *bcl-2* ARE transcript with different concentrations of ribozyme (100 nM, 10 nM, 1 nM) or sterile water (control). As a control of the ribozyme specificity, the IGFR transcript was incubated with 100 nM ribozyme or with sterile water (control). The denaturation step was followed by addition of sterile water and TrisHCl 1M pH 8. Tubes were heated to 90°C for 1 minute and cooled to 37°C. The reaction started adding 1 µl of $MgCl_2$ 100 mM in all the tubes (except the control sample of ARE without $MgCl_2$) and was performed for 1 hour at 37°C. In order to stop the reactions , 20% deionized formamide and 250 mM EDTA were added and the samples were heated to 90°C for 2 minutes, chilled on ice. Then 2.5 µl of gel loading buffer (50% glycerol, 1 mM EDTA pH 8.0, 0.25% Bromophenol Blue, 0.25% Xylene Cyanol) and 12.5 µl of deionized formamide were added. The samples were heated to 70°C for 5 minutes and chilled on ice, then they were electrophoresed in a 5% polyacrilamide gel, 8 M urea and transferred on a nylon filter (Amersham) by a Semi-Dry transfer cell (Biorad, Hercules, CA). The substrate and the degradation product were revealed with the digoxigenin system (Hoffmann-La Roche).

Determination of ribozyme steady state parameters. Kinetic constants were determined from Eadie-Hofstee plots[23] obtained from initial velocities with multiple turnovers done with UTP-labeled substrate. 3.3 nM and 10 nM ribozyme stock solutions

were used following the procedure described above. The kinetics was performed for 3 hours. The degree of RNA cleavage under standard conditions was determined after autoradiography followed by scanning laser densitometry. Kinetics constants for the cleavage of synthetic RNA were obtained by plotting the observed cleavage rate K_{obs} against the quotient of K_{obs} over the ribozyme concentration $[E]_g$ according to the following equation:

$$- \ln (FracS)/t = - K_m K_{obs} / [E]_g + K_{react}$$

The reaction rate K_{obs} equals the negative natural logarithm of the remaining fraction of synthetic RNA (FracS) divided by the reaction time *t*. The negative slope represents the K_m value, and the intercept of the regression line with the ordinate gives the maximal reaction rate K_{react} under single turnover conditions.

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