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Advances In Brief

Inhibition of the Translocated *c-myc* in Burkitt's Lymphoma by a PNA Complementary to the *Eμ* Enhancer[1]

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Abstract

In Burkitt's Lymphoma there is an up-regulation of the *c-myc* oncogene caused by its translocation from chromosome 8 to chromosome 14, often close to the *Eμ* enhancer of the immunoglobulin heavy chain locus (IgH). In Burkitt's Lymphoma cells, a peptide nucleic acid complementary for a specific unique *Eμ* intronic sequence selectively blocked the expression of the *c-myc* oncogene under *Eμ* control but not of other *c-myc* alleles. This Peptide Nucleic Acid also inhibited IgM expression in B cells. The finding that PNAs specific for a regulatory noncoding sequence can block gene expression has important conceptual and practical implications.

Introduction

PNAs[3] are nucleic acid analogues where the natural backbone is substituted with uncharged pseudopeptidic 2-aminethylglycine units (1, 2). PNAs are resistant to nucleases and proteases and bind with high affinity and specificity to complementary RNA and DNA. Moreover, PNAs can invade duplex DNA and hybridize with complementary sequences successfully displacing the natural cDNA strand (1–4). Because of these properties, PNAs have been used as antisense or anti-gene reagents in a number of experimental systems (1, 2, 5, 6). Unfortunately, PNAs enter the cells by endocytosis but are sequestered in the endosomal compartment before they can reach the cell nucleus (3). In previous *in vitro* studies, this problem was overcome by linking a NLS to PNAs, which facilitates nuclear localization (2–6). BL in three cells with a translocated and up-regulated *c-myc* oncogene were treated *in vitro* with a PNA specific for the second exon of the *c-myc* oncogene (PNAmycwt) linked to a NLS. In these conditions, there was a rapid down-regulation of *c-myc* and a consequent inhibition of two functions controlled by the *c-myc* itself: proliferation and apoptosis (3). However, because a PNA complementary to a coding DNA sequence can block the expression of both the translocated oncogene and the proto-oncogene also active in normal cells, some toxicity resulting from inhibition of the normal proto-oncogenes was to be expected if PNAmyc was to be used for therapeutic purposes. Therefore, a new strategy aimed at selectively inhibiting the expression of the translocated *c-myc* was needed.

In BL cells, the translocation of *c-myc* from chromosome 8 causes its juxtaposition to the *Ig* loci on chromosome 14 or less frequently on chromosome 2 or 22 (7). The *Ig* locus on chromosome 14 is a complex structure comprised of groups of different *V*, *D*, *J* and *CH* gene segments. After recombination in ontogeny, a unique *VDJ-CH* gene structure, which encodes the complete *IgH* chain, is formed (8) in the single mature B cells. Regulatory elements are positioned within the *IgH* chain locus, the most relevant being the *Eμ* intronic enhancer (Eμ) between *JH* and *Cμ* and the 3′ *IgH* locus elements downstream the *C* gene loci (9). *Eμ* contributes to *VDJ* recombination, switch recombination, and *H* chain gene transcription (10–12). Although all of the *Eμ* structures contribute to the regulation of these phenomena, a limited number of sites are necessary to guarantee the enhancer functions (13). These motifs, named *μA*, *μB*, *μC* in humans, bind to the Ets-1, PU.1, AML-1 factors, respectively (13), and are generally referred in their overall as minimal or core *Eμ*. In BL, because of the t(8;14) translocation, the *c-myc* oncogene is positioned near the *Eμ* intronic enhancer sequence (7), and the proximity of the *Eμ* enhancer is believed to be responsible for the up-regulation of *c-myc* expression (7, 9, 14). On the basis of these premises, we tried to determine whether the binding of specific PNAs to the *Eμ* core sequences would cause selective down-regulation of the translocated *c-myc* while leaving unaffected the expression of the *c-myc* genes not under *Eμ* influence (13).

Materials and Methods

PNA Synthesis. PNAs were manually synthesized under license of PerSeptive Biosystem using solid-phase peptide synthesis by Boc chemistry. First, the peptidic NLS (PKKKRKV) was synthesized followed by the PNA sequence portion. All compounds were purified by reverse phase high-performance chromatography on a Shimadzu LC-9A preparative high-performance chromatography equipped with a Waters X18μ Bondapack column (19 × 300). Mass spectra of each compound were obtained in the positive ion mode using a single quadrupole mass spectrometer HP Engine 5989-A equipped with an electrospray ion source and confirmed by electrospray iontrap mass spectrometry.

A PNA unique and complementary to the 3′-5′ strand of the Eμ sequence that encompasses the *μA* and *μC* elements was synthesized [PNAEμwt; GenBank accession no. X00253.1, bases 136–157: GCAGGAAGCAGGTCACCG-NLS]. Other PNAs included: PNAEμscr, CAGTCGGCAGCGGACAAG-NLS; PNAEμmut, (GCAGGAAGCA**A**GTCACCG-NLS$_{1mut}$; GCAGG**G**AGCAA**G**TCACCG-NLS$_{2mut}$; by PDTC, Rockefeller University, New York, NY); G**T**AGGAAG**T**AGGTCA**T**CG-NLS$_{3mut}$; PNAEμcomp, CGGTGACCTGCTTCCTGC-NLS. A *c-myc* anti-gene PNA (PNAmycwt) (GenBank accession no. X00364, bases 4528–4544, II exon TCAACGTTAGCTTCACC-NLS) as well as PNAmycmut, T**T**AACG**C**TAGCTT**T**ACC-NLS

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[3] The abbreviations used are: PNA, peptide nucleic acid; NLS, nuclear localization signal; Ig, immunoglobulin; AML-1, acute myelogenous leukemia 1; EMSA, electrophoretic mobility shift assay; PI, propidium iodide; BL, Burkitt's Lymphoma; Eμ, Eμ intronic enhancer; PNAEμwt, PNA complementary for a unique Eμ sequence; PNAEμscr, PNA with same base composition as PNAEμ but in randomized order; PNAEμmut, PNAEμwt with a point mutated sequence but unchanged pyrimidine purine ratio; PNAEμcomp, PNA specific for the complementary strand targeted by PNAEμwt; PNAmycwt, PNA c-myc anti-gene; PNAmycmut, PNAmycwt trimutated.

were synthesized as reported previously (3). None of the PNAs matched any known human gene sequence.

Cell Cultures. Two BL cell lines (BRG and RAMOS) and an EBV carrying B-cell line (Cor3) were used, originated, and kept in culture as described previously (3). PNAs (stock solution 1 mM in H_2O) were added directly to the culture medium (RPMI 1640; Seromed-Biochrom KG, Berlin, DE) at a 10 μM final concentration that was found to be optimal in titration tests (Ref. 3 and data not shown).

EMSA Analysis. The ^{32}P end-labeled oligonucleotide *E*μ DNA probe (TIBMolbiol s.r.l., Genoa, Italy) was incubated with or without 10 μg of nucleoprotein extract from EBV-positive Cor3 lymphoblastoid B-cell line in the presence of 1 μg of poly(dI-dC)·(dI-dC) (Sigma-Aldrich), 200 mM NaCl, and 20% glycerol for 15′ at room temperature. The reactions were resolved on a polyacrylamide gel. EMSA was also run in the presence of cold μA and μC DNA competitors (10 μM, 30 μM, 100 μM, and 1 mM) or of different concentration (1, 3, 10, and 30 μM) of the PNAs. Supershift EMSAs were carried out by incubating ^{32}P-labeled oligonucleotide *E*μ DNA alone or with nuclear extracts in the absence or in the presence of antibodies specific for Ets-1 and AML-1 (respectively, N-276: sc-111 and N-20: sc-8563; Santa Cruz Biotechnology Inc., Santa Cruz, CA). An irrelevant anti-NFKB p50 antibody was used as negative control (H-119: sc-7178; Santa Cruz Biotechnology, Inc.).

Run-Off Transcription Assay. Newly transcribed *c-myc* mRNA was quantified by run-off transcription assays as described previously (3, 15).

Cultured BRG and RAMOS cells were resuspended at 10^8 cells/ml in 200 μl aliquots in the presence of RNA Guard (500 units; Amersham-Pharmacia Biosciences, NJ) and permeabilized by addition of lysolecithin (Sigma-Aldrich). Equal cell aliquots were exposed to the various PNAs or to medium alone for 30′ at 37°C. ATP, CTP, GTP, and subsequently [α^{32}P] UTP (Amersham-Pharmacia Biotech, I) were added. Transcription was allowed to proceed for 30′ at 37°C. Total [α^{32}P] RNA was purified from each sample (16) and hybridized to filters where the DNA probes (custom made by TIBMolbiol s.r.l) for *c-myc* (bases 4831–4860, GenBank accession no. X00364), *G3PDH* (bases 633–662, GenBank accession no. NM_002046), and *Ig* μ chain (bases 451–480, GenBank accession no. M 28074) were slot-blotted and cross-linked. The amount of newly transcribed *c-myc*, μ *chain*, and *G3PDH* mRNAs in each sample was autoradiographically detected and analyzed.

Northern Blot. The RNA was purified from cells cultured with PNAs for 18 h as previously reported (3), size separated on an agarose-formaldehyde gel, transferred to Hybond TM -N + nylon membrane (Amersham, UK), hybridized with [^{32}P]-GTP random primed-labeled probes (kit; Boehringer Mannheim, Mannheim, Germany), and autoradiographed. The *c-myc* probe used was a 1.5-kb DNA fragment containing the II *myc* exon (Amprobe; Amersham, UK). The β-*actin* probe was cloned by reverse transcription-PCR from a noncorrelated cell line (β-*actin* AN X00351; PCR primers: base 2334, 5′-CTCACCCTGAAGTACCCCATCG-3′; base 1097, 5′-CTTGCTGATCCACATCTGCTGG-3′). The *c-myc* mRNA was expressed as percentage of the unsusceptible β-*actin* gene specific activities.

Western Blot. Western blot analysis was performed on purified nuclear proteins as described previously (3). Briefly, total cellular proteins were

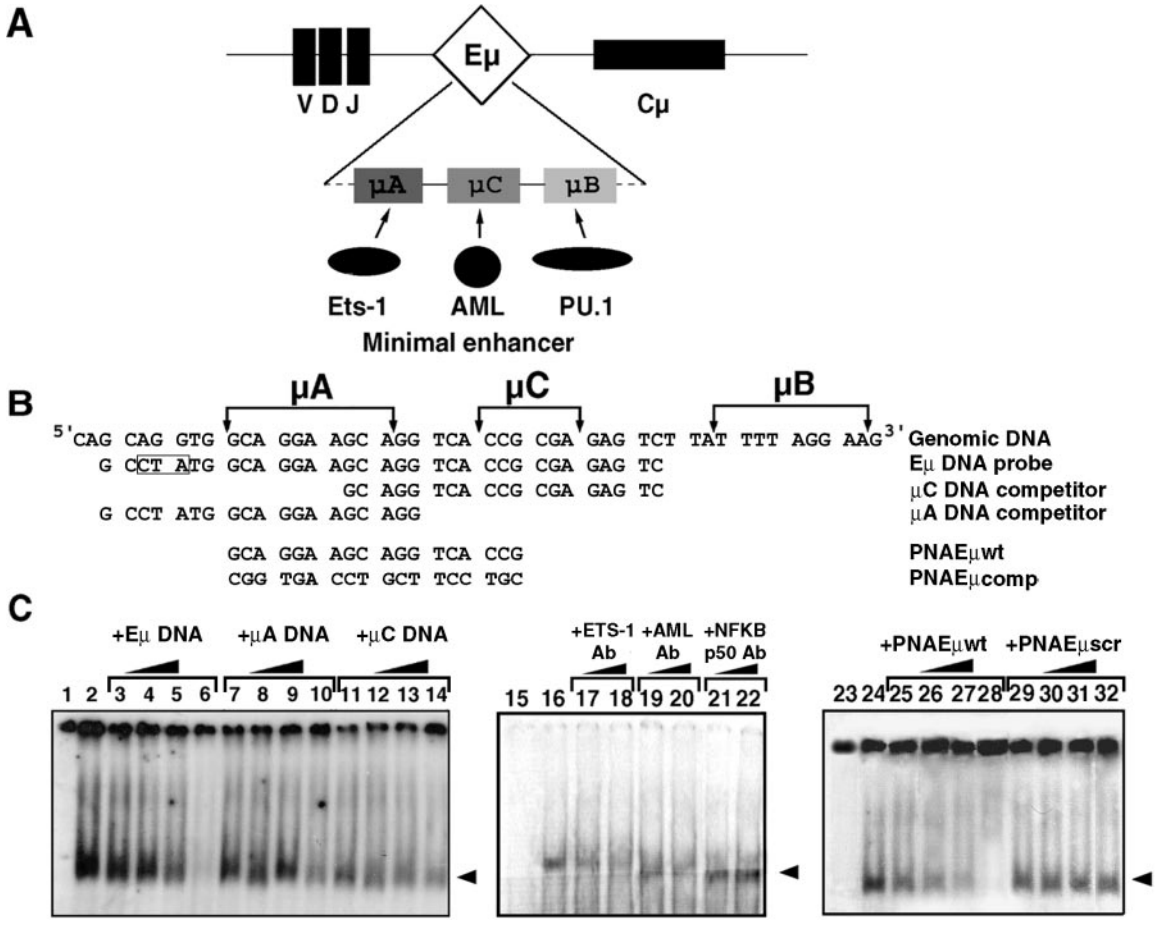

Fig. 1. PNAEμwt prevents binding of specific factors to *E*μ enhancer motifs. *A*, schematic representation of the *E*μ enhancer structure on chromosome 14. The *E*μ core elements are reported in details together with their specific nuclear factors. *B*, sequences of the *E*μ enhancer DNA probes and of the PNAs used in the EMSA test. Only the 5′-3′ DNA strands are shown. The *box* in the *E*μ DNA probe indicates a modification of the original sequence introduced to prevent the potential binding of nuclear factors different from Ets-1 and AML-1. The PNAEμwt was complementary to the 3′-5′, whereas PNAEμcomp was complementary to 5′-3′ strand of the *E*μ sequence. Both PNAs encompassed the μA and μC elements. *C*, EMSA was carried out using the radiolabelled *E*μ DNA probe (*Lanes 1*, *15*, and *23*), a mixture of nuclear extracts from Cor3 cell line with the radiolabelled *E*μ DNA probe in the absence (*Lanes 2*, *16*, and *24*) and in the presence (*Lanes 3–14*) of the indicated cold DNA competitors or specific antibodies to the involved nuclear factors (*Lanes 17–20*) and an irrelevant antibody to NFKB (*Lanes 21–22*; at a concentration of 200 ng or 1 μg/30 μl), or different amounts of PNAs, PNAEμwt (25–28) or PNAEμscr (*Lanes 29–32*): 1 μM, *Lanes 25* and *29*; 3 μM, *Lanes 26* and *30*; 10 μM, *Lanes 27* and *31*; 30 μM, *Lanes 28* and *32*). In this panel, the actual autoradiographic data of the electrophoretic separations of the mixtures described are presented. The shift in the gel run, caused by formation of specific complexes, is indicated by an *arrow*.

solubilized in urea lysis buffer electrophoretically separated on acrylamide SDS gels and then transferred to a nitrocellulose membrane (Hybond C-extra; Amersham). The membrane was stained with anti-myc antibody (9E10; Calbiochem, San Diego, CA) or anti-H2B antibody (FL-126: sc-10808; Santa Cruz Biotechnology, Inc.) followed by an alkaline phosphatase-conjugated goat antirabbit IgG (Sigma). MYC concentrations (from triplicate experiments) were expressed as ratio to the histone H2B protein.

Evaluation of Cell Growth, Cell Cycle, and Apoptosis. The variations in the growth curves of cell lines treated with PNAs was determined by cell counts (3). Cell cycle analysis was performed by PI staining in hypotonic solution and flow cytometry (FACSort; Becton-Dickinson, San Jose, CA; Ref. 3). Cell viability was evaluated by PI staining of intact cells in isotonic solution followed by flow cytometry (3).

BRG and RAMOS cells were incubated with different PNAs or medium for 12 h and exposed to a $F(ab')_2$ fragment of a rabbit antibody specific for human IgM(α-μAb) coupled to polyacrylamide beads (Irvine Scientific, Santa Ana, CA) as previously reported (5) or to a $F(ab')_2$ of an irrelevant antibody (data not shown). The cells were cultured for an additional 48 h, and apoptosis induced by cross-linking of surface IgM was evaluated by PI staining of cells nuclei in hypotonic solution and flow cytometry (17).

Results and Discussion

Inhibition of the Binding of Nuclear Factors to DNA by PNAs. The capacity of specific PNAs of inhibiting the binding of nuclear factors to the *E*μ DNA sequence was investigated by a gel shift assay. Fig. 1 shows a typical experiment and also illustrates the schematic structure of the *E*μ sequence as well as the binding sites of the majors nuclear factors (Fig. 1*A*). We used a DNA probe differing from the genomic sequences by the boxed bases in Fig. 1*B*. This figure also reports the cold DNA competitors used for inhibition experiments. Although all of the DNAs used were in the double-stranded conformation, only the 5′-3′ sequence is depicted. The figure also shows two PNAs complementary to the double-stranded DNA of the *E*μ minimal enhancer: PNAEμwt complementary to the 3′-5′ and PNAEμcomp complementary to 5′-3′ strand. Fig. 1*C* demonstrates that the addition of nuclear extracts from the lymphoblastoid cell line Cor3 caused a shift in the radiolabelled ^{32}P *E*μDNA probe in the gel assay (Fig. 1*C*, *Lanes 2*, *16*, and *24*). The shift was prevented by the addition of cold *E*μDNA probe (Fig. 1*C*, *Lanes 3–6*) or of specific DNA sequences identical to the μ*A* (Fig. 1*C*, *Lanes 7–10*) or μ*C* (Fig. 1*C*, *Lanes 11–14*) sequences of the *E*μ. The supershift assay demonstrated that specific antibodies inhibited the binding of Ets-1 (Fig. 1*C*, *Lanes 17* and *18*) or of AML-1 (Fig. 1*C*, *Lanes 19* and *20*). An irrelevant antibody to NFKB p50 (Fig. 1*C*, *Lanes 21* and *22*) had not such effect. These results are consistent with previous observations (13) and confirm the capacity of the test system of measuring the binding between the *E*μ sequences and the relevant factors. The presence of PNAEμwt (Fig. 1*C*, *Lanes 25–28*), but not of PNAEμscr (Fig. 1*C*, *Lanes 29–32*), had an inhibitory effect in the shift assay that resulted in a substantial decrease in the intensity of the radiolabeled bands. PNAs with a sequence that differed from PNAEμwt by 1–3 bases (PNAEμmut) failed to inhibit the binding of the nuclear factors to the *E*μDNA probe (data not shown). PNAEμcomp (Fig. 1*B* and data not shown) caused inhibition in the shift assay, although this was lower than the one induced by PNAEμwt.

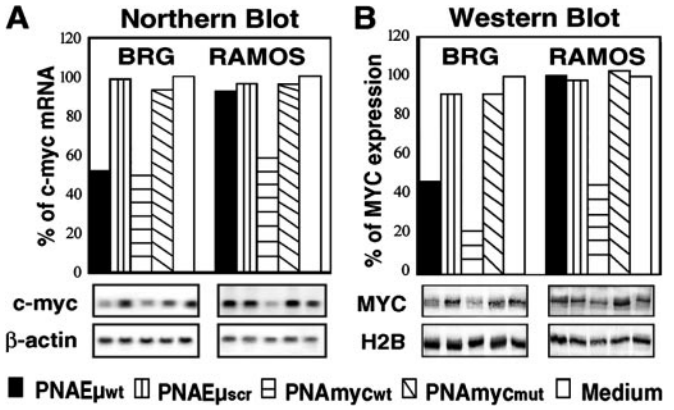

Fig. 3. Assessment of *c-myc* down-regulation by PNAEμw in viable BL cells in culture. *A*, *c-myc* mRNA expression. RNA was extracted from cells cultured for 18 h in the presence of the indicated PNAs and analyzed by Northern Blot with *β-actin* or *c-myc*-specific probes. *c-myc* mRNA concentrations were determined from the autoradiographic signals and expressed as percentage of mRNA of the unsusceptible *β-actin* gene. MYC protein expression. MYC protein was detected by Western blot in cells cultured for 48 h in the presence of the indicated PNAs. MYC concentrations are here normalized (NIH image) to histone H2B concentrations and reported as percentages. The data in *A* and *B* are representative of three experiments.

Ability of PNAEμwt of Inhibiting *c-myc* Transcription in BL Cells. Subsequently, we investigated whether PNAEμwt inhibited the transcription of the *c-myc* oncogene in BL cells, which express only the translocated *c-myc* and not the normal allele (18). For these experiments, two cell lines were used: BRG, where the breakpoint on chromosome 14 occurs in the *JH* region and the two translocated exons of *c-myc* oncogene are near the *E*μ enhancer sequence (19, 20), and RAMOS, where the breakpoint on chromosome 14 occurs in the switch μ region (21), and the three translocated *c-myc* exons are located in proximity of this site, whereas the *E*μ sequence is translocated on chromosome 8 (Fig. 2*A*). BL cells were permeabilized with lysolecithin, exposed to the different PNAs, and *c-myc* or μ *chain* mRNA production was measured in a run-off transcription assay.

In this test, PNAEμwt (but not PNAEμscr or PNAEμmut) substantially inhibited the transcription of *c-myc* mRNA in BRG but not in RAMOS cells (Fig. 2*B*). The inhibition was similar to the one induced by PNAmycwt (3). PNAEμwt (but not PNAEμscr) inhibited the transcription of the *Ig* heavy μ *chain* in RAMOS and BRG cells (Fig. 2*B*), as well as in EBV-positive lymphoblastoid B-cell lines, whereas the production of γ or α mRNA remained unaffected (data not shown). Likewise, PNAEμwt failed to inhibit α *chain* mRNA synthesis in BRG-A cells (a switch variant of BRG cells that produce

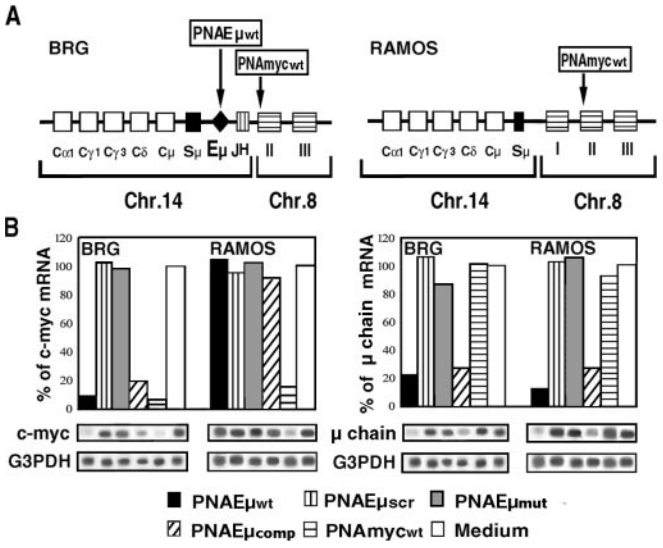

Fig. 2. Down-regulation of *c-myc* and μ-*chain* transcription by PNAEμwt in permeabilized BL cells. *A*, schematic representation of the topography of the *c-myc* rearrangement in BRG and RAMOS cells. The specific targets for the PNAs used are also indicated. *B*, newly transcribed *c-myc* and μ-*chain* mRNA were evaluated by a run-off transcription assay on permeabilized BRG or RAMOS cell lines treated with the indicated PNAs. The autoradiographic response of the quantitative slot-blot of each mRNA preparation is shown in the lowest panels. The histograms report the effect of the various PNAs on *c-myc* and μ *chain* transcription calculated as percentage of an internal control: the nonresponsive gene *G3PDH*. This experiment is representative of three tests.

only IgA; Ref. 20; data not shown). PNAEμcomp inhibited *c-myc* transcription in BRG (and not in RAMOS cells) although at a lower extent than PNAEμwt. Furthermore PNAEμcomp, although blocked significantly IgM expression, was less efficient than PNAEμwt in both BRG and RAMOS cells (Fig. 2*B*).

Down-Regulation of *c-myc* Expression in Viable BRG Cells by PNAEμwt. Next we investigated whether PNAEμ, like all the PNAs used here, was linked to NLS peptide and could therefore enter the cell nuclei and block *c-myc* expression in viable BL cells *in vivo*. BL cells were incubated with the various PNAs *in vitro* and tested for both mRNA transcription and MYC protein expression. As shown in Fig. 3, *c-myc* mRNA transcription and MYC translation in BRG cells were inhibited by PNAEμwt at levels similar to those observed with PNAmycwt treatment. PNAEμwt treatment was not effective at inhibiting *c-myc* expression in RAMOS cells.

Inhibition of Proliferation and Apoptosis of BRG Cells by PNAEμwt. Next, we tested whether the proliferation of BRG cells caused by *c-myc* deregulation was blocked by PNAEμwt. As shown in Fig. 4*A*, the cell cycle analysis of cells cultured with various PNAs for 72 h showed that PNAEμwt caused a reduction of the S and G_2 + M phases of the cell cycle in BRG cells comparable with the one induced by PNAmycwt treatment. PNAEμcomp, although able to

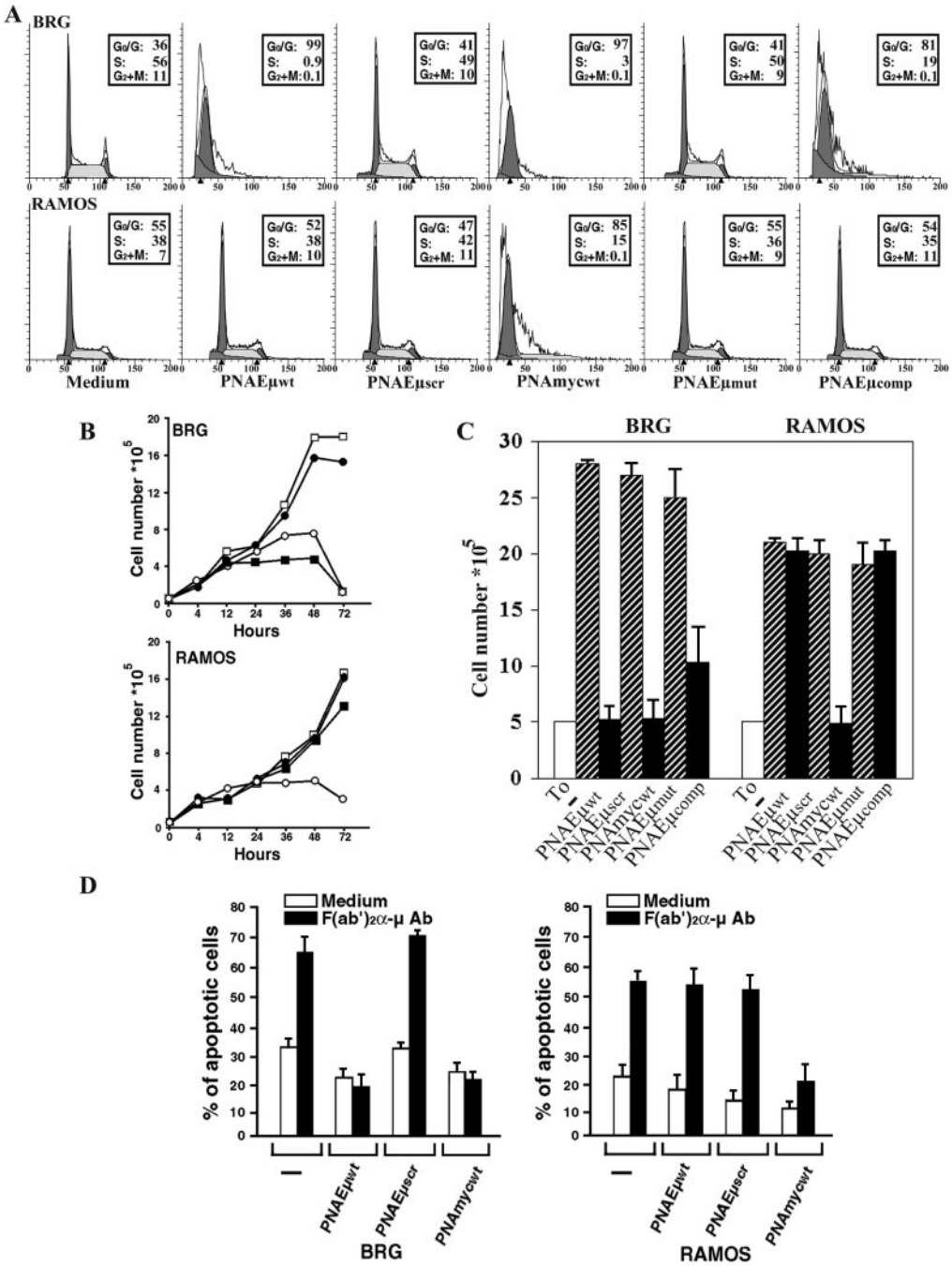

Fig. 4. Growth and apoptosis inhibition of BL cell line by PNAEμwt. *A*, flow cytometric analysis of the cell cycle in BRG or RAMOS cells cultured with the indicated PNAs for 72 h. *B*, growth curve of BRG and RAMOS cells treated with different PNAs (□, medium; ■, PNAEμwt; ●, PNAEμscr; ○, PNAmycwt). *C*, cell numbers detected in BRG or RAMOS cell cultures treated with the indicated PNAs for 72 h. Identical numbers (5×10^5) of cells were plated in the different cultures and the absolute number of cells determined. *D*, apoptosis of BL cells exposed to F(ab′)$_2$ α-μ antibody or to medium alone in the presence of the indicated PNAs. Apoptosis was measured by PI staining of permeabilized cells and flow cytometry.

inhibit the cell cycling capacity, was consistently less efficient than PNAEμwt. In contrast, PNAEμscr or PNAEμmut were ineffective. As expected, because of the topography of the t(8;14) chromosomal translocation, PNAmycwt only could inhibit the cell cycle in RAMOS (Fig. 4*A*). Fig. 4*B* shows the growth curves of BRG and RAMOS cells in the presence of the various PNAs, whereas Fig. 4*C* reports the total cell number detected in the cultures treated with the different PNAs for 72 h. Again inhibition of cell growth by PNAEμwt was only observed in BRG cells.

The up-regulation of *c-myc* renders BL cells apoptosis prone, particularly when surface IgM is cross-linked by an appropriate antibody *in vitro*. Here, we investigated whether PNAEμwt, by inhibiting *c-myc* expression, also prevented apoptosis. BL cells were treated with different PNAs for 12 h and subsequently exposed to an $F(ab')_2\alpha$-μAb. As shown in Fig. 4*D*, PNAEμwt treatment substantially inhibited the apoptosis of BRG (and not of RAMOS) cells in this setting.

Concluding Remarks. This study demonstrates that DNA regulatory sequences can be targeted with specific PNAs. The presence of the PNAs, by blocking the binding of specific nuclear factors, results in the inhibition of the regulatory functions of the DNA sequences as shown by the down-regulation of *IgM* synthesis and the expression of the *c-myc* oncogene under *E*μ control. Interestingly, although PNAs complementary to both the sense and the antisense strand of the *E*μ sequence were able to inhibit both transcription of the μ *chain* and of the translocated *c-myc*, the PNA specific for the antisense strand (PNAEμwt) was more efficient than PNAEμcomp. This phenomenon is presently being investigated in depth, but it may suggest a preferential binding of nuclear factors to the antisense DNA strand. Other investigators have reached a similar conclusion when studying the blocking of the Ets binding by DNA methylation (22).

The present observations reinforce the hypothesis that the proximity of the *E*μ enhancer increases *c-myc* oncogene expression in BL, presumably through the same mechanism required for μ *chain* expression (8, 12). These data are also of practical relevance. First, specific PNAs can be used to elucidate the potential regulatory role of certain genomic sequences, the function of which is still poorly understood. Second, they can be used as anticancer agents by targeting the regulatory sequences, which facilitate the expression of certain oncogenes. This approach seems particularly suitable for oncogenes that as a consequence of a chromosomal translocation have acquired new and unusual regulatory elements. The specific block imposed by PNAs on the regulatory sequences would not affect the expression of the normal corresponding proto-oncogene in nonneoplastic cells, and these agents should be less toxic *in vivo* than the PNAs targeting the oncogene sequences themselves. In the case of lymphoproliferative disorders, the *E*μ sequence appears to be a relevant common target because a variety of translocated oncogenes (*bcl-2*, *bcl-1*, *bcl-10*) may be under *E*μ control (7). Moreover, the hypogammaglobulinemia that would be likely a common side effect of this approach would not invalidate the application of this therapy because only IgM synthesis is affected by PNAEμwt.

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