Doctor's Thesis
学位論文

Molecular mechanisms of gene expression of the germinal center-associated DNA-primase GANP

胚中心B細胞選択DNAプライマーゼGANPの遺伝子発現制御機構

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Abbreviations

Ab	antibody
Ag	antigen
AMV	avian myeloblatosis
BCR	B cell antigen receptor
Blimp-1	B lymphocyte-induced maturation protein-1
β-gal	β-galactosidase
bp	base pair
cDNA	deoxyribonucleic acid complementary to messenger RNA
CD40L	CD40 ligand
ConA	concanavalin A
DTT	dithiothreitol
EBF	early B cell factor
EDTA	ethylene diamine tetraacetate
EMSA	electrophoretic mobility shift assay
ERK1/2	extra cellular regulated protein kinase 1 and 2
FCS	fetal calf serum
FDC	follicular dendritic cells
GANP	germinal center-associated DNA primase
GC	germinal center
Ig	immunoglobulin
Inr	initiator
JNK	Jun N-terminal kinase
Kb	kilobase(s)
LPS	bacterial lipopolysaccharide
mAb	monoclonal antibody

MAPK	mitogen-activated protein kinase
MCM	minichromosome maintenance protein(s)
μF	microfarad
Mut	mutant
ONPG	o-nitrophenyl-D-galactopyranoside
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI3K	phosphatidyl inisitol-3 kinase
PMA	phorbol myristate acetate
PMSF	phenylmethylsulfonylfluoride
5'-RACE	rapid amplification of the 5'cDNA end
SDS	sodium dodecyl sulfate
SSC	saline-sodium citrate
SV	simian virus
TD	T cell-dependent
TdT	terminal deoxynucleotidyl transferase
V	volt
WT	wild-type

Summary

Antigen stimulation induces rapid B cell proliferation for clonal expansion and differentiation to antibody-secreting cells in the germinal centers (GCs). The mechanism(s) that controls the proliferation and differentiation of antigen-specific B cells in the GC remains undetermined and is a particularly important issue as these cells undergo various molecular events, including somatic hypermutation in immunoglobulin (Ig) V-region genes, isotype switching, and affinity maturation of the antibody response, during their proliferation in the GCs.

GANP is a DNA-primase associated with MCM3 of the DNA replication complex and is up-regulated selectively in GC-B cells, and is proposed to play an important role in the DNA replication and cell proliferation through its ability to synthesizes DNA primers at the replication origin. To understand the molecular mechanism that controls the B lineage-associated and stage-dependent *ganp* gene expression, we isolated and characterized the 5' promoter region involved in the developmental stage-dependent expression of *ganp* gene using B lineage cells of various differentiation stages. Sequence analysis and the primer extension mapping have indicated that *ganp* initiates transcription at multiple sites from the TATAA-less promoter. Using the luciferase reporter gene assay, selective up-regulation of *ganp* expression was observed in the -737-bp (relative to the major transcription initiation site designated as +1) promoter region in B and plasma cell lines but was significantly low in pre-B and T cell lines. The deletion constructs displayed a gap decrease after shortening the region from –134- to –108-bp. Further narrowing and site-directed mutagenesis suggested the involvement of the PU.1 consensus sequence at –126-bp in the up-regulation of *ganp* expression. Further studies demonstrated that the protein component PU.1 complex bound to the consensus motif in *ganp* promoter region whose interaction is sequence-specific and is inhibited by anti-PU.1 antibody. Moreover,

introduction of *PU.1* cDNA enhanced the reporter gene activity in a sequence-specific manner in B cells, which is augmented by the stimulation with anti-CD40 antibody whereas the reporter construct with the mutated PU.1 site did not respond. This result clearly supported the previous observation that the stimulation by CD40 enhanced the expression of GANP DNA-primase in B cells both *in vivo* and *in vitro*. In addition, we found that a binding site for the cell cycle regulator E2F at –357-bp plays an enhancing role in the regulation of *ganp* promoter. These results demonstrate that the transcription factor PU.1 is involved in the regulation of stage-dependent expression of the germinal center-associated DNA primase GANP, probably by a cell cycle-associated mechanism undertaken by the E2F transcription factor.

1. Introduction

1.1. General introduction.

B cells are generated from haematopoietic precursor cells in the bone marrow and proceed to differentiate through successive stages accompanied by the rearrangement of immunoglobulin (Ig) genes and the acquisition of unique cell surface phenotypes. The B-cell lineage can be divided into three major stages of differentiation which have been largely defined by changes in the pattern of Ig gene expression. Pre-B cells represent the earliest stage in which the Ig μ heavy-chain gene is expressed. Subsequently, the Ig κ or Ig λ light-chain gene is expressed in B cells, which display Ig molecules on the cell surface. Pre-B and B cells maturate through the antigen-independent stages of B-cell differentiation. After encountering with antigen, and with the help of costimulatory factors and cytokines provided by T cells and other cellular components of the lymphoid organs, antigen-driven B cells further maturate in the peripheral lymphoid organs and terminally differentiate into antibody-secreting plasma cells. Such functional and structural changes in B cells during the differentiation are mainly due to down- or up-regulation of many stage-dependent transcription factors and the consequent effect on the B cell target genes. Especially for the terminal differentiation, many of the functional and cell surface receptor molecules need to be suppressed. Thus, the activation and subsequent proliferation including DNA replication and differentiation of mature B cells into plasma cells is a critical event within the B cell differentiation process, because it is associated with the affinity maturation of specific antibodies.

B cell activation and differentiation is regulated in part by the concerted action of various nuclear transcription factors required for the clonal expansion of antigen-specific B cells in GC in the lymphoid follicles of antigen-immunized animals and their differentiation into high affinity antibody-secreting cells, and the selection and

conversion of the affinity matured B cells into memory B cells. Despite the accumulating data regarding the molecular requirements for B cell activation and GC formation, such as the critical role for CD40-CD40L interactions and the developmental requirements for the tumor necrosis factors and their receptors, little is known regarding the mechanisms of generating somatic hypermutations in Ig genes, and differentiation to the memory cells or plasma cells. There are questions remained unanswered, for example; how the rapid cell growth necessary for clonal expansion is regulated, what is the mechanism for introducing somatic hypermutations in Ig genes during the clonal expansion, how the expression level of antigen receptors is down regulated in antibody-producing plasma cells, what is the turning point for the terminal differentiation of B cells into plasma cells. Thus, the GCs, where various molecular events involved in the B cell differentiation and maturation occur, come as the focal point to address these questions.

A novel B cell differentiation antigen, GANP, has been identified as a potentially critical molecule for the activation and differentiation of antigen-stimulated B cells in GC area. GANP up-regulated in GC B cells but not in T cells has unique functional domains which are considered to be involved in the regulation of DNA replication. Studying the transcription regulation of GANP gene in B lineage cells would be a key issue to understand the molecular mechanism required for the activation and differentiation of antigen-specific B cells in GCs.

1.2. Introduction to the present work.

1.2.1. Proliferation and differentiation of antigen-driven B cells and the associated molecular events in the GC.

B cell activation is initiated by antigen binding to surface immunoglobulin (sIgM), which signals naive B cells of resting state to enter the cell cycle. As most of the soluble antigens require T-cell help for effective B cell stimulation as a form of T

cell-dependent (TD) antigen (Ag), activated B cells with antigens can either differentiate to become plasmacytes or, alternatively, migrate to the secondary lymphoid follicles of the spleen and lymph nodes to form GCs and undergo clonal expansion with the help of follicular dendritic cells (FDC) and antigen-primed T cells (Rajewsky, 1996; Liu and Aprin, 1997; MacLennan, 1994). Ag-driven B cells first start proliferating and accumulate as large blast cells that rapidly move through the cell cycle, localize at the dark zone of GC area and further differentiate into small non-dividing centrocytes whose proliferation is arrested abruptly in the light zone of GCs (Foy *et al.*, 1994; Kelsoe, 1995). During the transition from rapidly proliferating centroblasts to non-dividing centrocytes, Ag-driven B cells undergo various molecular events for clonal expansion, affinity maturation, class switching from IgM to IgG, and the selection of B cells for further differentiation into long-lived memory B cells or terminally-differentiated antibody (Ab)-secreting plasma cells. Among these molecular events, the mechanism that controls the proliferation of Ag-specific B cells in the specified area of GCs remains undetermined.

Ag-driven B cells differentiate with expression of various cell differentiation markers as peanut agglutinin (PNA), GL-7, CD23, and CD21 but with reduced expression of cell surface B cell receptor (BCR)(Igarashi *et al.*, 2001; Shinall *et al.*, 2000; Mitsuyoshi *et al.*, 1997). GC-B cells proliferate with rapid DNA synthesis and expression of the cell-cycle-associated molecules as Ki-67, and proliferating cell nuclear antigen (PCNA) (Mitsuyoshi *et al.*, 1997; Kelman, 1997). During the differentiation of GC-B cells, various molecular events would appear to be associated with an effective Ab production, some of which are probably closely associated with cell-cycle progression that is inevitable for clonal expansion of Ag-reactive B cell clones. Some events also include somatic hypermutation in Ig V region genes (MacLennan, 1994; Storb, 1996; Wang *et al.*, 2000), isotype switching (Kawabe *et al.*, 1994; Zan *et al.*, 1999), and the arrest of cell proliferation before selection in the FDC

network (MacLennan, 1994: MacLennan *et al.*, 1997). Details of such molecular mechanisms, including clonal expansion and differentiation to the memory cell or plasma cell stage, somatic hypermutation, and class switching remain undetermined. Recent studies, however, suggested that these events occurring on Ig genes are not necessarily linked to the active DNA synthesis during DNA replication but might be associated with sterile transcription of Ig genes that appears after the end of DNA synthesis during cell cycle progression (Snapper *et al.*, 1993; Tierens *et al.*, 1999).

1.2.2. Differential expression of B cell specific genes is associated with the proliferation and differentiation state of the cell.

The B cell differentiation programme is a tightly regulated process, characterized by an ordered, sequential induction and extinction of specific sets of structural and regulatory gene products that are primarily controlled by a combination of tissue-specific and ubiquitous transcription factors. Based on their stage-specific expession pattern, B-lineage-specific genes can be classified into three groups (see Fig. 1). The first group represents genes expressed at very early stage of B cell development. Included in this group are λ5 and Vpre-B (Chen and Alt, 1993; Rolink and Melcher, 1993), the RAG1 and RAG2 (Chen and Alt, 1993; Landau *et al.*, 1984; Peterson *et al.*, 1984), and the TdT (Landau *et al.*, 1984; Peterson *et al.*, 1984) genes. The second group represents genes expressed at all stages of B cell differentiation, except for the late-stage terminally differentiated antibody-producing plasma cells. These includes transcription factors such as B cell-specific activator protein BSAP (Barberis *et al.*, 1990) and early B cell factor EBF (Hagman *et al.*, 1993), cytoplasmic proteins as Btk (Rawlings and Witte, 1995) and Blk (Dymecki *et al.*, 1990), as well as transmembrane proteins as CD19 (Tedder and Isaacs, 1989), CD40 (Stamenkovic *et al.*, 1989), Ig-α (Sakaguchi and Melchers, 1986; Hombach *et al.*, 1990), and Ig-β (Hombach

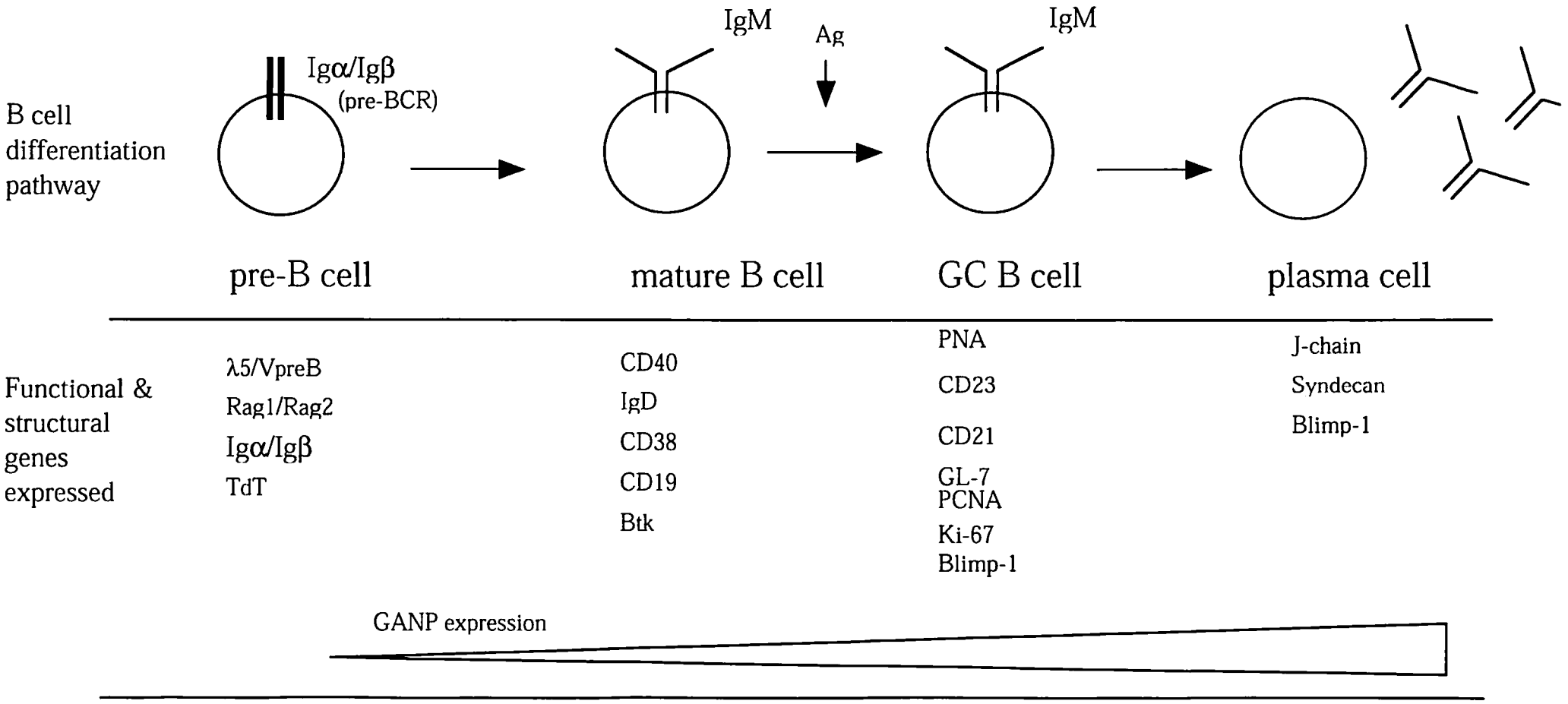


Figure 1: A diagram of B cell maturation and differentiation and the associated expression of the most important genes required for the proper differentiation program.

et al., 1990; Hermanson *et al.*, 1988). The third group represents genes that are specifically expressed after activation and further maturation of B cells, such as Blimp-1 (Turner *et al.*, 1994), Syndecan (Jalkmanen *et al.*, 1992 and the J chain (Koshland, 1985) genes. Most of these molecules are considered as proliferation and/or differentiation markers expressed at various stages of B cell maturation (see Fig. 1). Although functions of these genes have been characterized, the mechanisms involved in regulating their differential expression as well as the molecular events associated with their stage-specific expression remain largely unknown.

The GC-associated DNA-primase (GANP) was recently identified by our group as a differentiation antigen up-regulated selectively in the rapidly proliferating Ag-driven GC-B cells but not in T cells (Kuwahara *et al.*, 2000 and 2001; Abe *et al.*, 2000). GANP protein is hardly detected in normal lymphoid tissues and organs, but is up-regulated in GCs of Ag-immunized spleen and lymph nodes. GANP protein levels are nearly undetectable in resting primary spleen B cells, but is induced markedly by stimulation with anti-IgM Ab plus anti-CD40 monoclonal antibody (mAb), implying its stage-dependent up-regulation in B cells undergoing rapid proliferation and terminal differentiation (Kuwahara *et al.*, 2000).

1.2.3. Transcription regulation and B cell differentiation.

The correct differentiation and maturation of the B lineage cells from precursor cells in the bone marrow depends on the appropriate expression of stage- and lineage-specific genes (Reya and Grosschedl, 1998; Henderson and Calame, 1998). This is achieved by the interaction of transcription factors with promoter and enhancer elements (see Fig. 2). A variety of such transcription factors includes PU.1, NF-κB, Ikaros, Blimp-1, E2A, EBF, and BSAP (reviewed in Glimcher and Singh, 1999). One of such transcription factors shown to be essential for B cell development is the macrophage and B cell-specific transcription factor PU.1. PU.1 is an Ets family

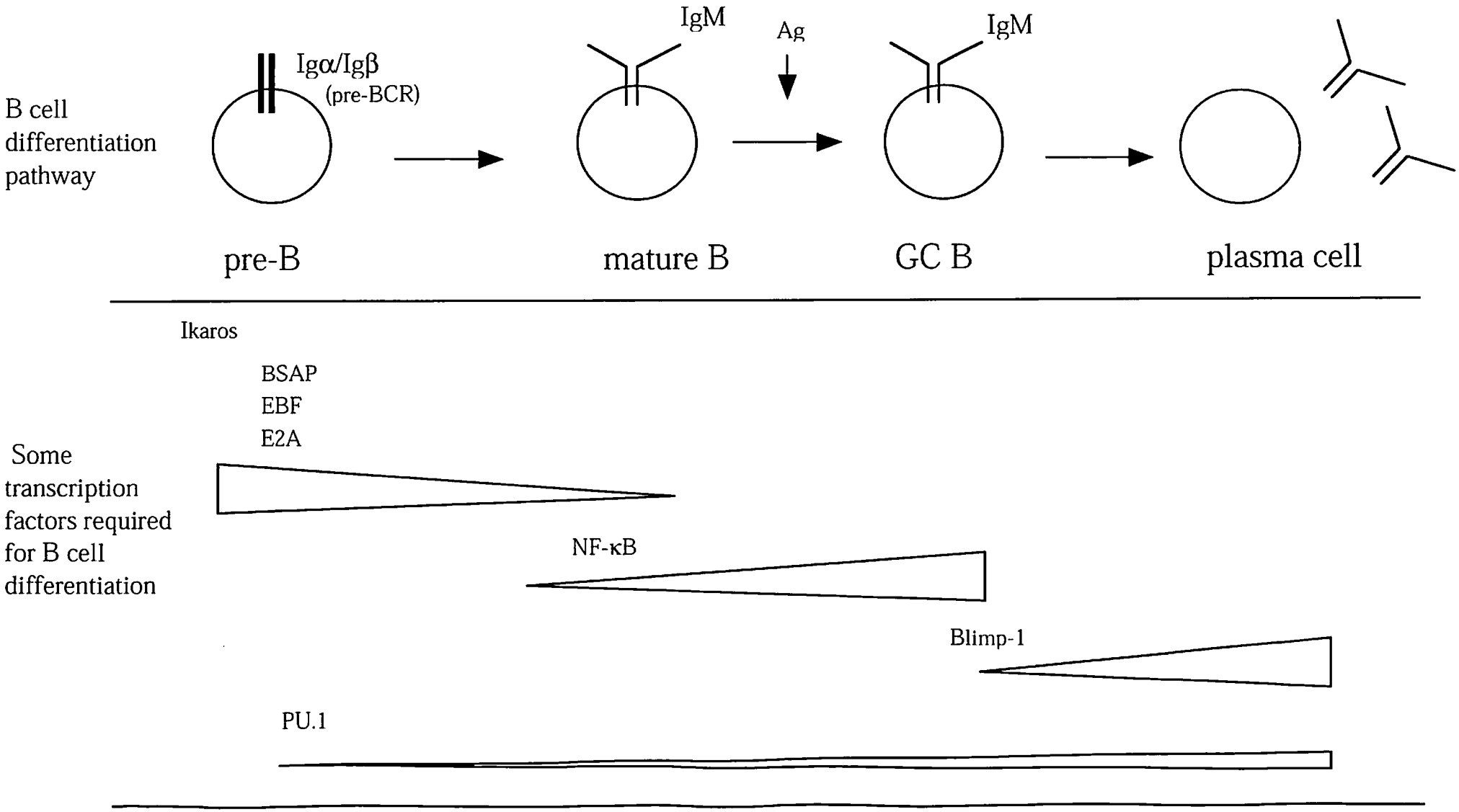


Figure 2: Expression boundaries of some transcription factors necessary for the development and differentiation of B cell.

transcription factor that is present in B-lymphoid cells and macrophages and binds to a purine-rich sequence that contains the central core 5'-GGAA-3' (Klemsz *et al.*, 1990). This family of transcription factors contains three functional domains, the highly conserved C-terminal DNA-binding domain (the Ets domain), the N-terminal transactivation domain, and the middle (PETS) domain which might be involved in protein-protein interaction (Moreau-Gachelin, 1994; Pongubala *et al.*, 1992).

1.2.4. GANP as a new DNA primase.

GANP protein binds to MCM3 through a domain identical to an MCM3-binding molecule Map80 that possesses an acetyltransferase activity for MCM3 (Abe *et al.*, 2000; Takei *et al.*, 2001). MCM3 is a member of minichromosome maintainence MCM proteins family that are abundant nuclear proteins involved in the regulation of genome replication. Recent studies of DNA replication in eukaryotic cells (Bell and Stillman, 1992; Donovan *et al.*, 1997; Takei and Tsujimoto, 1998; Vashee *et al.*, 2001) have demonstrated a requirement of a complex that recognizes the origin of DNA replication and the associated components MCM. The MCM complex required for DNA replication is composed of at least six identified components known as MCM2, 3, 4, 5, 6, and 7; some of which are phosphorylated during cell cycling (Lei *et al.*, 1997; Tanaka *et al.*, 1997; Jiang *et al.*, 1999). The prereplication complex (pre-RC) is formed at the end of mitosis, and its formation is initiated by the association of cdc6 protein with the origin recognition complex ORC, followed by the loading of MCM proteins onto the origin (Hateboer *et al.*, 1998). Cyclin-dependent kinases are thought to phosphorylate component(s) of the ORC, resulting in initiation of DNA replication (Hateboer *et al.*, 1998). Thus, loading of MCM complex with its associated protein(s) onto the chromosome could be seen as a limiting step in the onset of DNA replication, and a key issue in the initiation of DNA replication is to understand the molecular function and gene regulation of the molecules associated with the prereplicative

machinary assembled onto the replicating chromatin.

A number of DNA polymerases that are used for DNA synthesis and DNA repair pathways might be differentially expressed during periods of quiescence and proliferation (Winter *et al.*, 2000). DNA polymerase α, δ, and ε are mainly involved in DNA replication and are expressed prevalently in the proliferative S phase of the cell cycle (Zan *et al.*, 2001; Burgers, 1998). The well studied DNA polymerase α primase, which is crucial for creating RNA primers at all mammalian origins of replication and then synthesizing short stretches of DNA, was found to be expressed in the rapidly-dividing GC B cells but not in the resting B cells (Winter *et al.*, 2000). This indicates an important role for DNA polymerase α in rapidly proliferating B cells undergoing specialized DNA repair processes during heavy chain class switching of Ig constant region genes and somatic hypermutation of Ig variable region genes. However, because the DNA polymerase α is broadly expressed in various different tissues and is not restricted to GC regions, the DNA polymerase GANP complex that is selectively expressed in GCs would be important.

GANP bears a DNA-primase activity that is inducible by the phosphorylation upon Ser^{502} of GANP (Kuwahara *et al.*, 2001). The findings regarding the structure and the associated components of GANP suggested a role for GANP in the proliferation and DNA replication of lymphoid lineage cells. The up-regulation of GANP appears selectively in B cells stimulated with anti-CD40 mAb *in vitro* or in GC-B cells stimulated with TD-Ag *in vivo*, but it does not appear in the T cell area of GCs. These observations imply the selective up-regulation of GANP expression in B lineage cells undergoing maturation and differentiation into Ab-secreting cells for TD-Ags. The stage-associated expression of DNA primase GANP might be involved in maturation of Ag-driven B cells in secondary lymphoid follicles, which would imply a role for *ganp* in the DNA synthesis and replication of such rapidly proliferating cells.

1.2.5. Aim of the present study.

Most of the molecular events that take place in Ag-driven B cells in the GCs are critical for the proper B cell proliferation, differentiation, and the maturation of the antibody response. This study aimed to investigate the molecular mechanism(s) that control the selective up-regulation of *ganp* gene expression. To address this issue, we have undertaken the isolation and characterization of the *ganp* promoter region by employing molecular biology approaches to define and characterize the regulatory DNA sequences that control *ganp* expression. We studied candidate transcription factors that control the B lineage-associated and stage-dependent expression of *ganp* gene.

2. Materials and Methods

2.1. Routine laboratory techniques and materials.

Unless specified, most of the procedures described here were carried out according to the standard laboratory manuals; molecular cloning (Sambrook *et al.*, 1989) and Current protocol in molecular biology (Ausubel *et al.*, 1990). The commercially supplied materials and kits were used acoording to the manufacturers' instructions, as follow;

Extraction of plasmid DNAs: DNA purification Kit (Qiagen).

DNA purification: GeneClean, Glass Max DNA isolation matrix system (Gibco, BRL).

Southern blotting: Random primer DNA labeling kit (Takara) and [α-^{32}P]dCTP isotope (Amersham).

Host strains: *E. coli* strain K802 as a host for plaque formation, and XL1-Blue as a host for plasmid DNA propagation.

DNA subcloning: Restriction and modifying enzymes, T4 polynucleotide kinase, bacterial alkaline phosphatase, DNA ligation kits (all from Takara).

PCR and DNA sequencing: Taq polymerase (Takara), Fluorescent labeled primer sequencing kit (Amersham Biosciences), and ABI 373A DNA sequencer (Applied Biosystems).

2.2. Cellular techniques.

2.2.1. Cell lines and cell culture.

The mouse lymphoid cell lines pre-B cell (70Z/3, 18-81), mature B cell (A20, BAL17), plasma cell (X63, SP2/0), and thymus T cell (BW5147, EL-4) were as described previously (Sakaguchi *et al.*, 1988). They were maintained in a complete RPMI-1640 culture medium (Invitrogen) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Dainippon Pharmaceutical, Osaka, Japan), 100 μg/ml streptomycin, 100 units/ml penicillin, 2 mM L-glutamine (BioWhittaker, Walkersville, MD), and 50 μM 2-mercaptoethanol at 37 °C in a 5% CO_2 incubator.

2.2.2. Isolation of splenic B cells and thymocytes.

Single cell suspensions were prepared from spleens and thymi of 8 weeks age-matched C57BL/6 wild-type mice. The spleens and thymi were removed, washed with cold medium, and then put onto a mesh in a petri dish filled with cold medium. The cells were squeezed out of the spleen and the thymi by squashing them through a mesh with the plunger of a 5 ml syringe. In the case of the spleen cells, the erythrocytes were removed by resuspending the cell pellet in 10 ml culture medium plus 9 ml of lysis buffer containing 8 ml of solution I (0.83% NH_4Cl) and 1 ml solution II (0.17 M Tris.HCl, pH 7.6) at room temperature for 5 min. Cells were collected by centrifugation at 1500 rpm for 5 min, and then washed twice with cold culture medium. Purified spleen cells and thymocytes were resuspended and counted using a Neubauer hemocytometer after staining with trypan blue to exclude the dead cells, and then cultured in complete medium in 75 cm^2 culture flasks at a density of $1x10^6$/ml.

2.2.3. *In vitro* cell stimulation.

Purified spleen cells and thymocytes were cultured for certain periods of time

in the presence of LPS (10 μg/ml) and ConA (5 μg/ml), respectively. The mature B cell, A20, was transfected as described below and then stimulated with anti-CD40 mAb (LB429; 10 μg/ml) or PMA (10 ng/ml).

2.3. Molecular biology techniques.

2.3.1. RNA extraction.

For Northern blot analysis of PU.1 expression, total RNA was extracted from exponentially growing cells by Tizol Reagent (Life Biotechnologies) according to the protocol supplied with the kit. For primer extension analysis, poly $(A)^+$ RNA was extracted from the total RNA, which was first extracted as described above, by elution through Oligo(dT)-Cellulose column, followed by washing and ethanol precipitation (Amersham Biosciences).

2.3.2. DNA extraction.

Genomic DNA was extracted from the propagated phage clones by the plate lyzate methods (Sambrook et al., 1989). Briefly, the recombinant phage DNA from the positive clones was propagated in K802 competent cells onto a 15 cm LB/ 1.5% agar plates, overlaid with 0.8% agarose. Just before confluent lysis (before the plaques reach each other, ~ 9 h to overnight), the plaques were extracted by adding 9 ml of SM buffer (containing 100 mM NaCl, 8 mM MgSO4, 50 mM Tris-HCl, and 2% glycerol) without chloroform onto the top of the plate. The plates were agitated gently on a shaker for about 6 h at room temperature. The collected lysate was then transferred to a 15 ml Falcon tube and centrifuged for 20 min at 8000 rpm and 4 °C. The supernatant (~4 ml) was transferred to a fresh tube and this was followed by adding 50 μl of 10 mg/ml DNase I (to digest the exogenous DNA) and 5 μl of 10 mg/ml RNase A (to digest RNA). After gentle mixing, the tube was incubated in a water bath at 37 °C for 30 min. After adding

6 ml of polyethylene glycol/NaCl mix (20% w/v PEG /2.5 M NaCl), the lysate was mixed, left for 3 h on ice, and then centrifuged for 20 min at 4 °C. The phage pellet was resuspended in 500 µl SM, dissolved by pipetting and transferred into 1.5 ml microfuge tube. A 20 µl of 0.5 M EDTA (pH 8.0) and 5 µl of 10% SDS was added, mixed and incubated for 15 min at 60 °C. After adding 500 µl of the equiliberated phenol, the contents were mixed for 10 min using a rotator at room temperature, and then centrifuged for 5 min at 15000 rpm and 4 °C. After repeating this step twice, 500 µl chloroform (without isoamylalcohol) were added and mixed by rotating the tube for 10 min. After centrifugation, the supernatant was mixed with 40 µl of 3 M NaOAc (pH 5.2) and 1.1 ml of 99% ethanol and incubated for 30 min at –80 °C. The tube was centrifuged for 10 min at 15000 rpm and 4 °C. The DNA pellet was then washed with 70% ethanol, dried (not completely) at room temperature, dissolved in 100 µl of 1x TE buffer, and kept at 4 °C for few months.

2.3.4. DNA sequencing.

The PCR was carried out using the Big Dye Terminator sequencing kit (ABI, Applied Biosystems). The following components were mixed on ice in a total volume of 20 µl.

Template DNA (0.5 µg/µl)	1µl
Distilled H_2O	10 µl
Big Dye Terminator mix	8µl
Primer (10 pmol/µl)	1µl
	20 µl

The PCR reaction was carried out in a PC-700 thermal cycler (Astec, Fukuoka, Japan) using the following conditions:

5 min at 96 °C for 1 cycle (initial denaturing), followed by 30 cycles of 10 sec at 96 °C (denaturing), 5 sec at 50 °C (annealing), and 4 min at 60 °C (elongation). The PCR

product was purified by ethanol precipitation, dissolved in template suppressor reagent, denatured at 95 °C for 2 min, immediately kept on ice, and then loaded in a capillary sequencer.

2.3.3. Subclonning PCR.

The PCR reaction was carried out in a total volume of 25 µl as follow:

Template DNA (50 ng/µl)	1µl
Distilled H_2O	16 µl
dNTP mix (2 mM)	2.5 µl
10x PCR buffer	2.5 µl
5' primer (10 pmol/µl)	1 µl
3' primer (10 pmol/µl)	1µl
DNA polymerase (5 U/µl)	1 µl
	25 µl

The following PCR programme was applied:

10 min at 94 °C (initial denaturing), followed by 35 cycles of 30 sec at 94 °C (denaturing), 30 sec at 50 °C (annealing) and 2 min at 72 (elongation).

The product was purified by ethanol precipitation, dissolved in 1x TE, digested with the relevant restriction enzymes, and purified by separation onto agarose gel in 1x TAE buffer and then recovered and subcloned into the relevant plasmid vector.

2.3.5. DNA radiolabeling.

2.3.5.1. By random priming reaction.

The cDNA probe used for the genomic library screening was radiolabeled using a random primer DNA labeling kit (Takara) as follows:

Template DNA (20 ng/µl)	1 µl
Random primer (kit)	2 µl

Distilled H2O	11 µl
	14 µl

The components were mixed in an Eppendorf tube, denatured by boiling for 3 min and immediately chilled on ice for 5 min. This was followed by adding 2.5 µl of 10x buffer, 2.5 µl of dNTP mix, 5 µl of [α-^{32}P]dCTP, and 1 µl of Exo-free Klenow fragment DNA polymerase I (all included in the kit). The polymerase was added last, and the reaction components were mixed and immediately incubated for 15 min at 37 °C. The labeled probe was then purified onto a Sephadex G-50 column prewashed twice with 1x TE/0.1% SDS buffer, eluted with the same buffer, ethanol precipitated by incubation at –80 °C for 30 min, pelleted and dissolved in 1x TE. The probe was either kept at –20 °C for overnight or denatured and used immediately for the hybridization reaction.

2.3.5.2. By kinase reaction:

The synthetic primer used in the primer extension mapping as well as the synthetic double strand oligonucleotides used in the electrophoretic mobility shift assay was end-labeled by a kinase reaction using the T4 polynucleotide kinase kit (Takara) as follows:

Oligonucleoted DNA (10 pmol/µl)	1 µl
10 x kinase buffer	1.5 µl
[γ-^{32}P]ATP	5µl
T4polynucleotidekinase (10 U/µl)	1µl
Distilled H2O	6.5µl
	15 µl

The reaction was incubated in a heat blocker for 30 min at 37 °C, and then purified by a Sephadex column and ethanol precipitated as described above, except that 1 µl of 20 µg/µl glycogen was added during the ethanol precipitation to increase the DNA yield.

2.3.6. Genomic library screening.

To isolate the 5'-flanking region of the *ganp* gene, a BALB/c mouse genomic library in the bacteriophage λ vector EMBL-3 was screened according to a standard procedure (Sambrook et al., 1989) with *Escherichia coli* strain K802 as a host for plaque formation. Briefly, approximately 10^6 phage clones were screened with the most 5'-end of the *ganp* cDNA (1.1-kb *Bam*HI-*Asp*718 fragment) radiolabeled probe. The probe was radiolabeled with [α-^{32}P]dCTP (Amersham Biosciences) by random primer extension. Nylon filters were prehybridized for 1 hr at 65 °C in a buffer containing 100 μg/ml sonicated salmon sperm DNA, and then hybridized overnight at 65 °C by adding the denatured probe to the same prehybridization buffer. After hybridization, the filters were washed 4 times with 2xSSC and 0.1% SDS for 5 min at room temperature, washed twice with 1x SSC and 0.1% SDS for 15 min at 65 °C, and finally washed twice with 0.1x SSC and 0.1% SDS for 10 min at 65 °C. The filters were dried at room temperature and exposed to an x-ray film with an intensfying screen for 2 days at -80 °C. After four rounds of screening, four independent clones containing slightly different-sized inserts named λG-2/3, λG-16, λG-23, and λG-25 were selected and the phage DNAs were isolated by the plate lysate method (Sambrook *et.al.*,1989) and analyzed and compared as described below.

For restriction mapping, the purified phage DNAs along with aliquots of genomic DNA extracted from the liver of BALB/c mouse, were digested with the restriction endonucleases *Bam*HI, *Hin*dIII, *Eco*RI, *Nde*I, *Xba*I, and *Sac*I under single digestion conditions and then separated on a 0.8% agarose gel. The gels were stained and photographed. The DNAs were then transfered to nylon membranes. As probes, five cDNAs (including the one used for the library screening) encompassing the entire *ganp* gene cDNA were used. Prehybridization, hybridization, washing and autoradiography were performed as described above.

2.3.7. DNA subcloning and sequencing.

Appropriate genomic DNA fragments recovered from the longest phage clone λG-25 (~17-kb) were subcloned in the vector pBluescript II KS (+) (pBS) and partially sequenced using the dideoxy chain termination method and ABI 373 A DNA sequencer. By comparing genomic and cDNA sequence data, we identified a 6.38-kb flank region as the putative promoter region, which extends from the *Xba*I site (in the middle of exon I) to the *Eco*RI site further upstream. A 6.38-kb *Eco*RI-*Xba*I fragment and 1.38-kb *Eco*RI-*Xba*I fragment was subcloned both in pBS and pUC19 plasmid vectors and designated as pBS/6.38-kb, pBS/1.38-kb and pUC19/1.38-kb construct. Most of the promoter constructs were then drived from these three subclones. The promoter sequence and consensus nucleotide motif analysis were performed using the DNasis software (Hitachi, Tokyo, Japan).

2.3.8. Reporter constructs for *ganp* promoter region.

To analyze the *ganp* promoter activity, a series of 5'-deleted fragments of the putative *ganp* promoter region were generated by subcloning using standard restriction enzymes and PCR methods and named according to their sizes relative to the major transcription start site (designated as +1). All genomic fragments were inserted at cloning sites of the promoterless/enhancerless luciferase reporter vector pGV-B (Toyo INK. MFG., Tokyo, Japan) upstream from the luciferase gene. The –737-bp reporter construct was generated by digestion of the pBS/1.38-kb construct with *Hin*cII. The *Hin*cII fragment was recovered and ligated in forward orientation into the *Sma*I sites of pGV-B vector. To make –637-bp construct, the *Pst*I-*Hin*cII fragment (637-bp) was recovered from pBS/1.38-kb construct, ligated into the correspoding sites of PBS vector, recovered again by *Sma*I/ *Xho*I digestion, and then subcloned into the same sites of pGV-B vector. The pBS/1.38-kb construct was digested with *Acc*I to remove *Acc*I fragment from its 5'-upstream. The DNA was recovered, self-religated, and then digested with

*Kpn*I/*Sac*I. This fragment (397-bp) was then recovered and subcloned into *Kpn*I/ *Sac*I sites of pGV-B vector to generate the –397-bp construct. The –264-bp reporter construct was made by recovering *Bam*HI-*Xba*I fragment (264-bp) from pBS/1.38-kb construct, ligating it into *Bam*HI/*Xba*I sites of pUC19 vector, recovering it again as *Sac*I-*Hin*dIII fragment, and then subcloning it into the same sites of pGV-B vector. The *ganp* promoter fragments of -168, -134, -108 and -54-bp as well as the reporter constructs harbouring E2F consensus sequence of the *ganp* promoter (-376-bp E2Fwt and –376-bp E2Fmut) were generated by PCR using the 1.38-kb DNA fragment in pBS as a template and sense and antisense primers containing *Sac*I site and *Hin*dIII site, respectively. The synthesised fragments were then digested with *Sac*I/*Hin*dIII and subcloned into the corresponding sites of pGV-B vector. The *E2F1* expression vector was described previously (Fujita *et al.*, 1999). All inserted genomic DNA fragments representing the 5' flanking sequence have 5'-ends extending varying distances upstream of the major transcription start site (designated as +1) and their 3'-ends are located immediately downstream of the major transcription initiation site. All constructed fragments were analysed by restriction enzyme digestion and sequencing to verify the proper insertion and orientation.

The pGV-P construct in which the luciferase gene is regulated by the SV-40 early promoter (PicaGene, Toyo, INK. MFG.) and the pSV-β-galactosidase construct (Promega, Madison, WI, USA) in which the β-galactosidase (β-gal) gene is controlled by the SV-40 promoter and enhancer, were used in the transient transfection experiment as a positive control (to measure the maximum reporter gene activity) and as an internal control (to normalize the transfection efficiency).

2.2.9. Site-directed mutagenesis.

Mutations in the E2F and PU.1 binding sites were generated using oligonucleotide primers carrying point mutations. The E2F consensus sequence 5'-

TTTGGGGC-3' was changed into 5'-CGATGTAC-3'. The primers used were: -377-bp E2FmutF, 5'-GGGAGCTCTAAGGCAAGTGGCGATGTACGGTTGCAATG-3' and –377-bp E2FmutR, 5'-GGAAGCTTGGAGGGAGGAGCAAGTCCGCCAGATA-3'. Note, the same point mutation was introduced into the E2F probe used for gel shift assay. The nucleotides 5'-GGAA-3', which are critical for PU.1 binding, were changed into 5'-AATT-3' as indicated by the underlined sequence in the primers shown below. The primers used were: -134-bp PU.1MutF, 5'-GGGAGCTCGAGAGCCAATTGCCGGA-3'; and –134-bp PU.1MutR, 5'-GGCAATTGGTCTCTGGGGTG-3'. Mutant luciferase constructs were generated by PCR and were confirmed by sequencing. For the construct of the -264-bp PU.1Mut, the DNA fragment of -134-bp PU.1Mut was joined into the construct by subcloning procedure. CD72 probe was prepared as 5'-GACCTTCTTCCTCTTTTATGACTT-3' according to the previous report (Ying *et al.*, 1998). Underline indicates the PU.1 binding site with opposite orientation.

2.3.10. Preparation of nuclear extract.

Nuclear extracts were prepared from lymphoid cells by minor modifications of the protocol described previously (Dignam *et al.*, 1983), and all steps were carried out at 4 °C. Briefly, exponentially growing ($2x10^8$) cells were harvested and washed with phosphate-buffered saline and the cell pellet was resuspended in 5 volumes of hypotonic buffer (10 mM Hepes [pH7.9], 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM phenylmethylsulfonyl floride (PMSF), 0.5 mM dithiothreitol (DTT), and 2 μg/ml each leupeptin, pepstatin, and aprotinin. The cells were centrifuged for 5 min in a Beckman JS-4.2 rotor (2475x g) and the packed cells were resuspended in 3 volumes of the same hypotonic buffer, kept on ice for 10 min, and then lysed by 15-20 up and down strokes in a Dounce homogenizer type B. Nuclei were pelleted for 15 min in the same centrifuge at 3300x g and then resuspended 1/2 volume of low salt buffer (20 mM Hepes [pH7.9], 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.2 mM

PMSF, 0.5 mM DTT, and 2 μg/ml each leupeptin, pepstatin, and aprotinin). This step was followed by addition of 1/2 volume (relative to the volume of the nuclear pellet before adding the low salt buffer) of high salt buffer (the same as low salt buffer except that KCl concentration was increased to 1.2 M). The nuclear proteins were extracted on ice for 30 min by gently shaking the tube. Following centrifugation for 30 min in Tomy RS-205 centrifuge (Hercules, CA), the nuclear extract (supernatant) was aliquoted, frozen immediately in liquid nitrogen, and stored at –80 °C. The same protocol was used to prepare nuclear extract from stimulated cells except that for primary spleen cells and thymocytes, the dialysis step was performed in a specially manipulated Eppendorf tube.

2.3.11. Transient transfection and reporter gene assay.

Transfection of all cell lines was conducted by electroporation using the Bio-Rad Gene Pulser II (Bio-Rad). Cells (1 x 10^7) were harvested and resuspended in 0.5 ml of the complete culture medium. Equimolar amounts of plasmids were added relative to 10 μg of the pGV-B luciferase reporter construct. One μg of pSV-β-gal construct was added as an internal control for the transfection efficiency. Cells were incubated with DNA for 5 min at room temperature in a 0.4-cm cuvette and then electroporated at 950 microfarads with 280 V (70Z/3), 300 V (A20), 270 V (X63), or 320 V (BW5147). After 5 min at room temperature, cells were transferred to culture dishes containing 10 ml of the complete culture medium and incubated at 37 °C for 48 h. Luciferase assay was performed using a luciferase assay kit (PicaGene) as described (Maeda et al., 1998). Cells were harvested, lysed in 400 μl of 1x lysis buffer (LCβ, TOYO INKMFG.) and protein extracts were prepared for luciferase and β-gal assays. For luciferase reaction, 20 μl of the cell lysate were mixed with 100 μl of luciferase substrate (PicaGene) cantaining 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 20mM N-Tris(hydroxymethyl) methylglycine, 1.07 mM $(MgCO_3)_4Mg(OH)_2\ 5H_2O$, 2.67 mM

$MgSO_4$, 0.1 mM EDTA, 33.3 mM DTT, 270 mM coenzyme A, 470 mM luciferin, and 530 mM ATP. The light emissiom was immediately counted for 30 sec by a luminometer. Luciferase activities were normalized against the β-gal activities for transfection efficiency as described previously (Maeda et al., 1998). The β-gal assay was performed by mixing 50 μl of the cell lysate with 50 μl of 2x assay buffer containing 120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 2 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and 1.33 mg/ml ONPG. The reaction was incubated for 1 h at 37 °C, and the β-gal activity was then measured by spectrophotometry. Each transfection was repeated at least 6 times (three times in duplicate) using three preparations of DNA, and representative data are shown. The mouse *PU.1* cDNA, introduced into the mammalian expression vector pCMV (Stratagene), was co-transfected with increasing doses as shown in Figs. 7 and 8.

2.3.12. Electrophoretic mobility shift assay (EMSA).

Electrophoretic mobility shift assay was performed with double-strand synthetic oligonucleotides between –255-bp and –108-bp as well as the region covering the E2F consensus sequence of the *ganp* promoter (Fig. 3A). Complementary synthetic oligonucleotides were mixed at an equimolar ratio in TE buffer [pH 8], heated for 5 min at 80 °C, and then annealed by slowly cooling down to room temperature. The double-strand probes were end-labeled with [γ-^{32}P]ATP (Amersham Biosciences) by T4 polynucleotide kinase, purified through a Sephadex G-50 column and ethanol precipitated.

For the binding reaction, 15 μg of the nuclear extract, 1 μg of poly (dI-dC) as a nonspecific competitor, and 0.5 ng (~ 1 x 10^4 cpm) of the labeled probe were mixed in a buffer (total volume, 20 μl) containing 20 mM Hepes pH 7.9, 15% glycerol, 300 μg/ml bovine serum albumin, 50 mM KCl, 0.2 mM EDTA, and 0.5 mM DTT. Otherwise stated, binding reaction mixtures were incubated for 10 min at room temperature with a

molar excess of the unlabelled nucleotide probe as a specific competitor for the labeled probe. Binding reactions were carried out for an additional 20 min at room temperature and were analyzed on 5% nondenaturing polyacrylamide gels (acrylamide/ bisacrylamide, 40:1) containing 2.5% glycerol for 2 h at 150 V and 4 °C after pre-running for 1 h at 100 V (Moreau-Gachelin, 1994). After drying, the gels were exposed to X-ray film for 1-2 days at –80 °C. Both the gel and running buffer contained 45 mM Tris, 32.3 mM boric acid, and 1.25 mM EDTA, pH 8.3.

For Ab inhibition, 4 µl of Ab against the DNA-binding domain of PU.1 (Santa Cruz Biotechnology) was added to the binding reaction mixture for 15 min prior to the addition of the labeled probe.

2.3.13. Primer extension mapping.

A DNA primer (5'-TCCGCCAGATACGGCCACTGTC-3') complementary to mouse *ganp* sequence nucleotides from +42 to +21 was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (TOYOBO, Osaka, Japan), and then purified using a Sephadex G-50 column. The labeled primer (2 x 10^5 cpm) was mixed with 4 µg of poly $(A)^+$ RNA extracted from the myeloma cell line SP2/0 in 10 µl of 5 x hybridization buffer containing 1.5M Nacl, 50 mM Tris-Hcl (pH 7.5), and 10 mM EDTA (pH 8.0). After being denatured at 80 °C for 4 min and then annealed for 2 h at 50 °C, reverse transcription reaction was performed in a total volume of 50 µl by adding the primer-RNA hybrids (10 µl) to 40 µl of 1.25 x reverse transcription buffer containing 1.25 mM each dNTP, 12.5 mM DTT, 12.5 mM Tris-HCl (pH 8.4), 7.5 mM MgCl2, 40 units of AMV reverse transcriptase, and 40 units of RNasin for 30 min at 42 °C. The extended product was extracted by ethanol precipitation, dried and dissolved in 5 µl formamide loading buffer and directly analyzed on a 6% sequencing gel in parallel with a sequencing reaction of the genomic DNA (using the same primer) as a size marker.

Results

3.1. Cloning and structure of the 5'-flanking region of the mouse *ganp* gene.

To study the mechanism of GC-associated *ganp* expression, we isolated DNA clones from EMBL3 genomic DNA library of C57BL/6 with the mouse *ganp* (Kuwahara et al., 2000) cDNA probe. A clone (λg-25) carrying the terminal 5' region was analyzed by subcloning (Figs. 3A and 4), and the 1.39-kb region was sequenced (EMBL Accession number AJ318088). Previously, we determined the longest 5' end of the mouse *ganp* cDNA by the 5'-RACE method (Kuwahara *et al.*, 2000). To determine the transcription initiation site of the mouse *ganp* gene, we performed the primer extension analysis and identified one major transcription start site (Fig. 3A and 3B) and two other minor sites (data not shown). One of two minor sites was identified as the longest 5' site by the 5'-RACE method (Kuwahara *et al.*, 2000). Based on these findings, we tentatively concluded the major initiation site of the mouse *ganp* gene (as indicated by +1 in Fig. 3A and 3B).

In the region from –400-bp to the major transcription site, significant similarities were found to the consensus sequences of E2F (at -357), E-box (at -333), BSAP (at -235), PU.1 (at -126), and an initiator-like sequence (at -61). In addition, an obvious canonical TATAA or CCAAT box is not found in the 5'-flanking region of the mouse *ganp* gene.

3.2. Determination of the DNA promoter region responsible for the B cell stage-dependent expression of *ganp* gene.

The function of the putative promoter region was analyzed using the reporter construct containing the DNA fragment from –737-bp to +1 and the transfection into cell lines representing various stages as pre-B (70Z/3), mature B (A20), plasma (X63) cells and T lineage cells (BW5147). This region showed a high promoter activity in

mature B and plasma cells as A20 (71-fold) and X63 (68-fold) over the background conferred by the control luciferase vector but showed low activities in only 70Z/3 (6-fold) and BW5147 (11.5-fold) (Fig. 4). Using all the constructs, similar differential expression was obtained in another set of representative cell lines as pre-B (18-81), B (BAL17), and T cells (EL-4)(data not shown).

Further truncation of the promoter region demonstrated that the region from -264 to –168-bp is involved in the stage-dependent regulation of *ganp* expression in comparison with the reporter construct of –168-bp. The deletion of this region resulted in a decrease in A20 (from 42-fold to 31-fold), but a slight increase in X63 (from 45- to 48-fold) and BW5147 (from 8- to 9-fold) (Fig. 4), suggesting a more complex regulation of this region in various differentiated cells. Additionally, the deletion from –134- to –108-bp caused a more marked decrease in A20 (from 39- to 19-fold) and X63 (from 48- to 16-fold) but again only a small decrease in BW5147 (from 8- to 6-fold) and 70Z/3 (from 2- to 1-fold). Truncation of the promoter region to -54-bp significantly decreased the promoter activity to a background level comparable with all cell lines tested. In addition, the construct with the reverse orientation of the -134-bp region did not show any promoter activity (data not shown). The data presented here clearly suggest that the 5'-flanking region up to –737-bp is partly involved in the B lineage-associated and stage-dependent expression of the *ganp* gene.

3.3. Detection of specific DNA-protein interaction sites in *ganp* promoter region.

To explore lineage-associated regulatory molecules involved in *ganp* expression, we conducted a series of EMSAs using radiolabelled oligonucleotides and nuclear protein extracts from the cell lines characterized above. Initially, we generated six oligo probes covering the entire region from –264- to –108-bp (Fig. 3A) and analyzed the DNA-protein interaction by EMSA. According to the initial results, two additional oligos namely oligo 7 and 8 were designed to cover the flanks of the oligos

that showed partial DNA-protein interactions; oligos 1&2 and 5&6.

A specific DNA-protein complex, GB-X, was detected by oligo 7. This complex was formed with nuclear extract derived from pre-B and mature B cells but not with extract from plasma (X63) or non-B cells (BW5147) (Fig. 5A). The same complex (albeit with lower intensity) was also detected in the nuclear protein extracts from normal mouse spleen B cells stimulated with LPS (Fig. 5B) but not from thymocytes (data not shown). This oligo contains a consensus sequence for the B cell specific activator protein BSAP. The binding of this complex is sequence specific as it was competed for by an excess of the unlabeled oligo 7 (lanes 10-13). Interestingly, GB-X disappeared in A20 cells after stimulation with PMA (lane 6), implying that the molecules involved in regulation of *ganp* expression through this sequence might be altered by the activation of mature B cells for differentiation into Ab-producing cells although the overall reporter activity has not changed much. Another specific DNA-protein complex, GB-1, was detected by oligo 8 with nuclear extract from B and plasma cells. The sequence corresponding to this element contains a binding site for the transcription factor PU.1.

3.4. Involvement of PU.1 in DNA-protein interaction at the ganp promoter.

Because the region (-134-bp to –108-bp) seems to be involved significantly in promoter activity and contains a consensus binding site for the transcription factor PU.1. we characterized the involvement of PU.1 as a DNA-binding protein in the regulation of *ganp* expression using A20 and X63 cells expressing *PU.1* mRNA (Nelsen *et al.*, 1993). The Oligo 8 probe detected two bands, one of which seemed to be non-specific because the cold probe could not efficiently inhibit its formation (Fig. 6A, lower band). The complex GB-1, detected with Oligo 8 covering the PU.1 consensus site (5'-GCGGAA-3'), could be inhibited by the cold 15-nucleotide probe (Oligo 9) containing the same PU.1 consensus site (Fig. 6A, upper band). The GB-1 complex is not detected by the

15-nucleotide probe with the mutation at the PU.1 site (Oligo 9Mut M1 and M2)(Fig. 6B), but the GB-1 complex, which is detected by the Oligo 8 probe containing the consensus PU.1 site, could not be inhibited by the cold probe with the mutation at the PU.1 site. The GB-1 complex was clearly inhibited by the cold probe with a known PU.1 consensus site at the promoter region of CD72 (Ying *et al.*, 1998) as a specific competitor, suggesting that the GB-1 complex contains the PU.1 protein (Fig. 6C). We also examined whether the formation of a GB-1 complex is affected by the addition of anit-PU.1 Ab. Specific Ab may cause a shift of the complex at the lower migration site on the EMSA or the disappearance from the gel by the inhibition of complex formation. The anti-PU.1 Ab used here showed rather an inhibition of the formation of the complex, indicating that the complex formed with the Oligo 8 sequence contains in part PU.1 protein (Fig. 6C). These results narrowed down the sequence element to the motif of the PU.1 consensus site. The contribution of the region with PU.1 consensus element to the function of the *ganp* promoter activity was obviously significant (Fig. 4). Lacking of this element (from –134-bp to –108-bp) in a reporter construct caused decreases of 51% (A20), 64% (X63), 55% (70Z/3), and 18% (BW5147) in the reporter gene activity. Collectively, these data suggest that the sequence element corresponding to the PU.1 consensus site is responsible for positive regulation of *ganp* promoter activity.

3.4. Effect of PU.1 in regulation of *ganp* promoter.

To determine the role of PU.1 in the regulation of *ganp* expression, we transfected the PU.1 expression construct together with a reporter construct containing the PU.1 site (-134/PU.1WT) or a construct harboring a mutation in the PU.1 site (-134/PU.1Mut). *PU.1* mRNA is expressed in B lineage cells (70Z/3, A20, and X63) but not in the T cell line BW5147 (Nelsen *et al.*, 1993), which was also confirmed before these experiments (data not shown). As shown in Fig. 7, the mutation introduced into

introduced into the PU.1 binding site resulted in a decrease of reporter activity in A20 and in X63 cells. This finding indicates that the PU.1 binding site is involved in the regulation of *ganp* promoter activity.

To investigate whether PU.1 protein is involved in the regulation of reporter activity, we introduced the cDNA construct that produces the PU.1 protein in the transfectants. Introduction of *PU.1* cDNA caused enhancement in reporter activity in a dose-dependent manner from 1 μg to 8 μg in A20 and X63 cells (Fig. 7). The mutation at the PU.1 site abolished the potential of enhancement in reporter activity even with the highest concentration of 8 μg.

The longer construct (-264-bp) carrying more motifs (Figs. 3 and 4) showed a similar enhancement in reporter activity in response to the exogenous *PU.1* introduction, but the activity was much higher than that of the shorter construct (Fig. 8). The negative effect of the mutation was not apparent in B cells presumably because of the much enhanced activity or of the cooperative regulation by the other elements in the longer construct; however, the mutant construct at the consensus PU.1 site could not augment the reporter gene activity in response to the *PU.1* introduction (8 μg). These results indicate that PU.1 is involved in the regulation of *ganp* gene expression through the PU.1 site (at –126-bp).

3.5. Stimulation-dependent induction of *ganp* promoter activity.

GANP protein expression in resting primary spleen cells is markedly increased by treatment with anti-CD40 mAb (Kuwahara *et al.*, 2000 and 2001; Abe *et al.*, 2000). Because the *ganp* promoter potentially regulates B cells by *in vitro* stimulation with anti-CD40 mAb, we examined whether this promoter region with the PU.1 site is a target of anti-CD40 signaling. We compared the reporter activity of a promoter construct (-134-bp and –264-bp) with or without the PU.1 element in A20 cells after stimulation (Fig. 9A). Based on non-stimulated controls, anti-CD40 mAb significantly

induced the luciferase activity by about 100%. The mutants of the PU.1 site could not show the up-regulation by anti-CD40 stimulation. Both the longer and shorter constructs showed similar results. On the other hand, stimulation with PMA induced a more increase in the promoter activity (Fig. 9B). However, deletion of the PU.1 element from the reporter construct did not alter the observed increase in luciferase activity due to PMA stimulation. These results suggest that the PU.1 site in the promoter region is involved in the anti-CD40-induced up-regulation of the *ganp* gene expression and also show that the DNA sequence element involved in the PMA-induced activation cascades lies further downstream within the –108-bp region.

In addition, the Rel/NF-κB transcription factors are critical regulators of the genes involved in diverse cellular processes including cell proliferation (Baeuerle and Baltimore, 1996), and stimulation through BCR and CD40 often relays signals resulting in activation and translocation of NF-κB to the nucleus, thereby activating gene transcription. IκB proteins control the transcriptional activity of NF-κB by retaining it in the cytoplasm, inhibiting its translocation to the nucleus and its subsequent interaction with the DNA. We aimed to investigate the effect of IκB inactivation on the *ganp* transcription. Addition of mutant IκB peptide, which can not suppress NF-κB activity into A20 cells transfected with a 1.38-kb promoter fragment and stimulated with CD40 did not affect the *ganp* promoter activity (data not shown). This result indicates that the CD40 signaling activates *ganp* promoter through pathway(s) other than the NF-κB pathway.

3.6. The transcription factor E2F positively regulates *ganp* expression.

The information regarding GANP, as its up-regulation in non-cycling centrocytes and association with MCM3, has suggested that *ganp* expression might be regulated in a cell cycle dependent manner. In the *ganp* promoter region, there is a consensus sequence potentially recognized by the E2F transcription factor (Fig. 3A).

To test whether this E2F-binding site contributes in the regulation of *ganp* transcription, a reporter construct containing the E2F site (-376/E2FWT) and a construct harboring a mutation in the E2F site (-376/E2FMut) were transfected into different cell lines. As shown in Fig. 10A, the mutation introduced into E2F binding site resulted in a decrease of reporter activity in A20 (from 52- to 43-fold) and X63 (from 72- to 53-fold) but only a small decrease in BW5147 (from 10- to 8-fold) compared with the wild-type construct. This finding indicates that the E2F binding site at nucleotide –364- to –357-bp is involved in the regulation of *ganp* promoter.

To investigate whether the E2F site in the *ganp* promoter is involved in specific DNA-protein interactions, a labeled oligonucleotide probe (oligo E2F) was incubated with nuclear extracts derived from B and T cell lines in the EMSA. Three E2F binding protein complexes are detected in pre-B and B cells while two complexes in plasma and T cells (Fig. 10B). The pattern of these complexes was very close to the one reported by other investigators (Chittenden *et al.*, 1993; Campanero *et al.*, 1999). The mutated E2F oligo did not show binding of the complex and a cold competition experiment using a molar excess of the unlabeled wild-type E2F oligo completely disrupted all the specific complexes formed (Fig. 10B, lanes 10-12). Interestingly, B cells stimulated for 48 h with the PMA showed a shift of most of the proteins with the lower mobility to a pattern somewhat similar to that of X63 cells but with different intensity (lanes 6 and 7). These results indicate that the E2F proteins specifically interact with the E2F site of the *ganp* promoter region.

E2F1 is the most studied member of the E2F family of transcription factors, and has been shown to activate transcription of several genes essential for DNA replication and S-phase entry, in particular PCNA and DNA polymerase α. To determine whether the *ganp* promoter can be activated or repressed by an exogenous E2F, a cotransfection assay was carried out using an expression plasmid encoding a full length *E2F1* cDNA and the reporter gene construct –376/E2FWT. Because E2F1, a

major partner of the retinoblastoma tumor suppressor (Rb) protein, exhibits some characteristic features of cell stage-specific activity (Gill and Hamel, 2000; Dicker *et al.*, 2000), we addressed whether the exogenous E2F1 will have an effect if any on the *ganp* transcription. Coexpression of *E2F1* activated *ganp* promoter-directed luciferase gene expression (Fig. 10C) in a dose dependent manner in plasma and T cells while in B cells the kinetic of such activation was different. With 4 μg of the *E2F1* expression plasmid, a maximum luciferase activity was observed 48 h post-transfection as increases of 180% in A20, 210% in BW5147, and 290% in X63 cells over the background conferred by the control reporter construct (-376/E2FWT) alone (assigned here a value of 1). This result suggests that the E2F site is involved in a direct activation of *ganp* promoter, at least in plasma and T cells. In the case of the B cells, it is less sensitive to exogenous *E2F* at doses less than 2 μg. The A20 cells require higher amounts of cDNA to significantly activate *ganp* promoter as it did in plasma or T cells. Also, considering the low affinity complexes formed with A20 cell extract (Fig. 10B, lane 4), we do not exclude a possible repressive effect by any pocket protein(s) in A20 cells. In this regard, a high level (specifically in B cells) of pocket or other E2F related proteins may sequester the binding of and/or the transactivation by E2F1. Collectively, however, the results from these analyses suggest that the E2F transcription factors bind to and transactivate *ganp* promoter, while *in vitro* proliferating cell lines may have a cell cycle associated regulation of *ganp* gene expression different from the normal ones of GC-B cells *in vivo*.

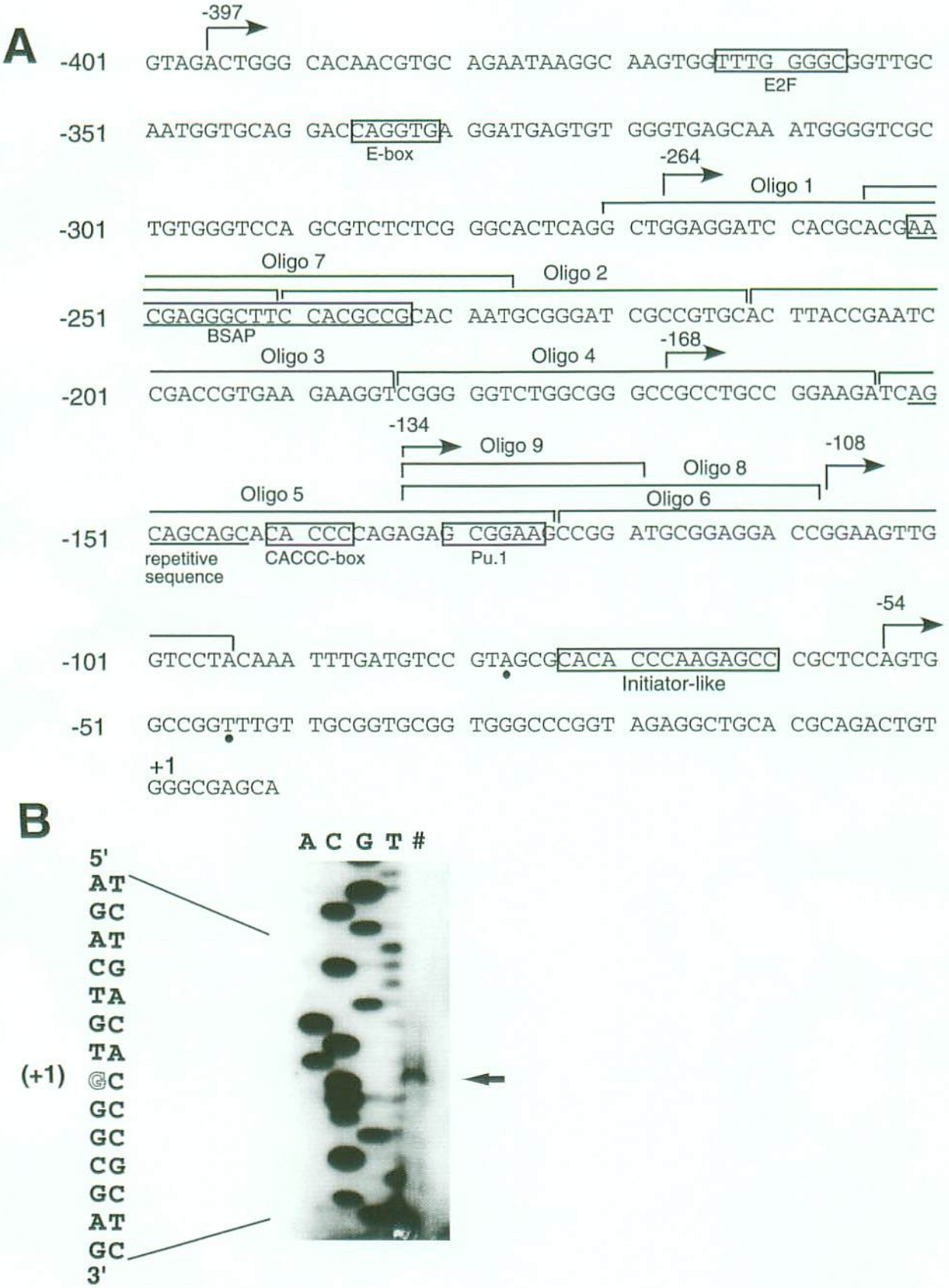


Figure 3 . Mouse *ganp* 5'-fanking sequence and transcription initiation sites. ***A***, Nucleotide sequence of the 400-bp fragment of the *ganp* promoter region. The major transcription start site has been numbered +1, and the minor start sites are indicated by *solid dots* below the sequence. *Curved arrows* show the 5'-end points of the reporter constructs. Several potential binding sites for transcription factors are *boxed*. Oligonucleotides used in EMSAs are shown. ***B***, Analysis of the transcription initiation by primer extension. An end-labeled oligo primer was annealed to poly (A)+ RNA extracted from myeloma cell line SP2/0, extended using AMV reverse transcriptase as described under "Experimental Procedures". The extended product (*lane* #) was analyzed on a sequencing gel together with a size marker. An *arrow* to the *right* indicates the major transcription initiation site. The two minor sites detected in this assay are indicated in ***A*** *by solid dots* below the sequence.

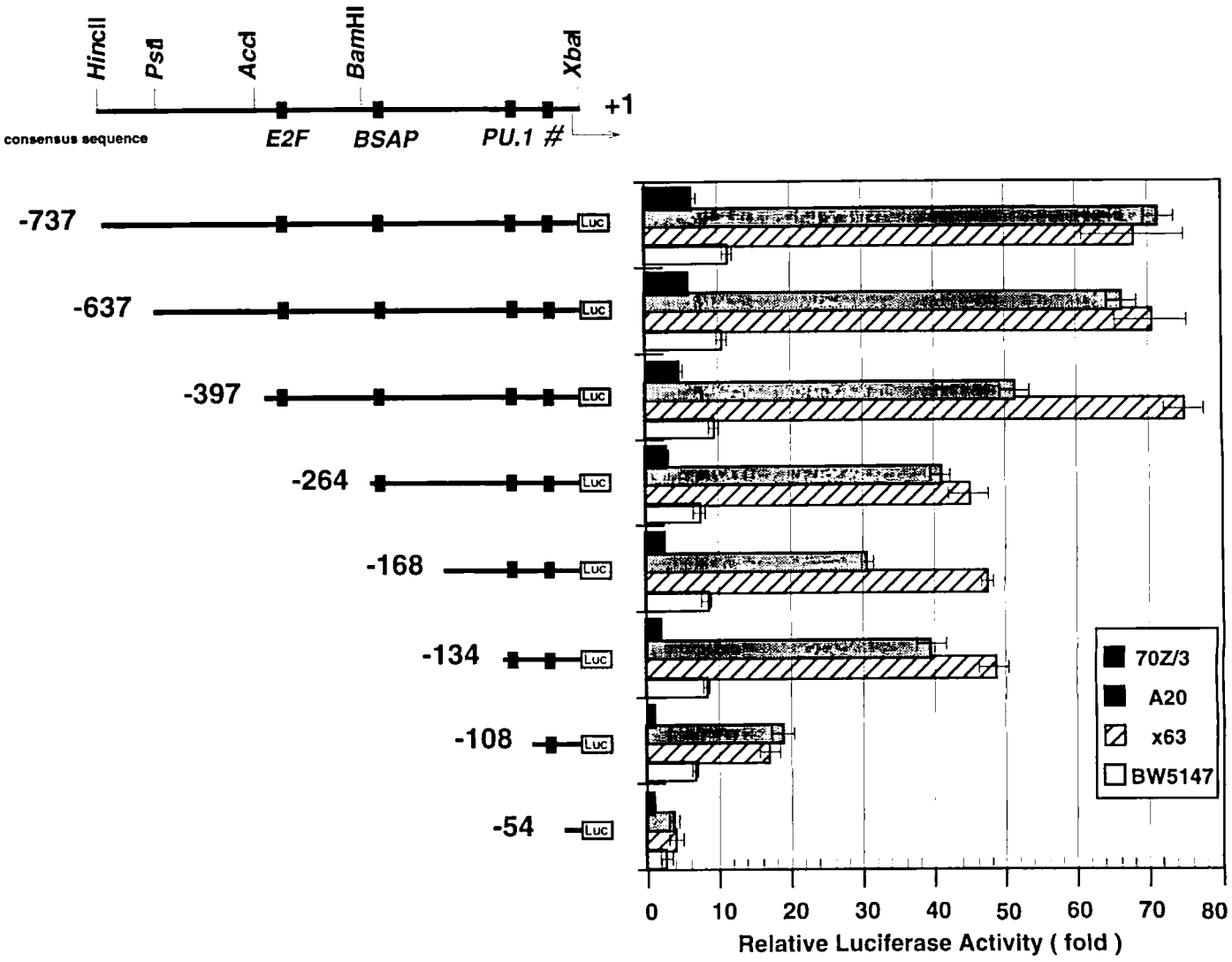


Figure 4. **Deletion analysis of the mouse *ganp* promoter.** *A* , a series of deletions from the *ganp* 5'-flanking sequence was inserted upstream of the promoterless/enhancerless luciferase gene. On the *left* is a schematic representation of *ganp*-based reporter constructs. All constructs are designated according to the insert size relative to the major transcription start site (designated as +1). The restriction enzyme sites used for promoter cloning and positions of three regulatory DNA motifs (*black boxes*) are indicated. On the *right*, the luciferase activity in 70Z/3, A20, X63, and BW5147 cells 48 h after transfection with the indicated promoter constructs is shown. The relative luciferase activity is expressed as fold activity above the background conferred by the promoterless/enhancerless control plasmid (pGV-B). Each histogram represents the value of luciferase activity for three independent experiments. Values shown are normalized by *b*-gal activity. The # mark refers to an undefined regulatory motif which is a downstream target of PMA signaling pathway.

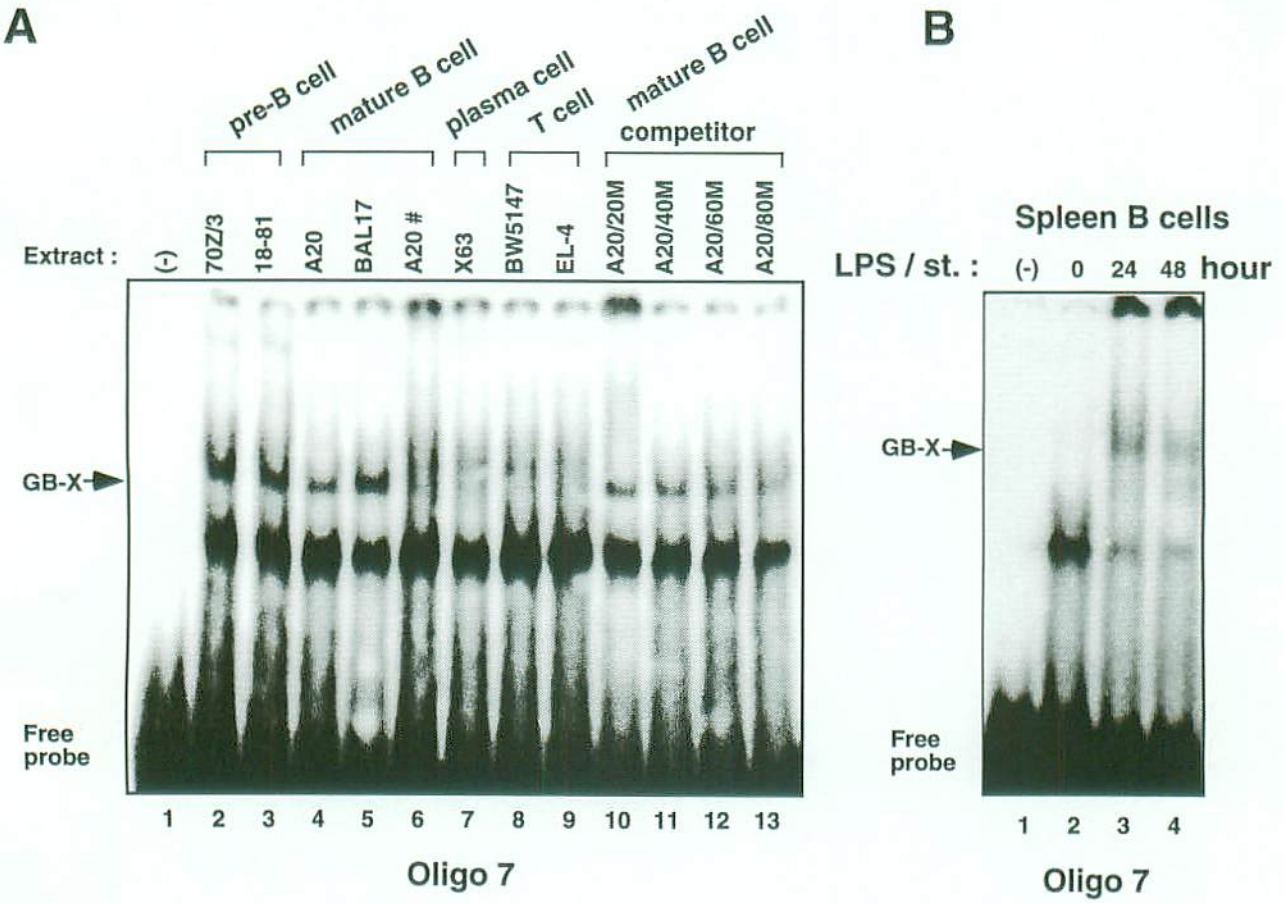


Figure 5. An early B cell-specific protein complex binds to the DNA fragment of the *ganp* promoter from nucleotide -255 to -227. ***A***, EMSA was performed with a double-stranded oligonucleotide probe (oligo 7) corresponding to nucleotides from -225 to -227. The probe was radiolabeled and incubated in the absence, or presence of 15 μg nuclear extract prepared from the indicated cell lines. In lane 6, extract from A20 cells stimulated with PMA for 48 h was used. In lanes 10-13, a molar excess of unlabeled probe was used as a specific competitor. Positions of the specific complex and free probe are indicated. Amounts of cold competitor are shown as molar excesses. ***B***, EMSA was conducted also using oligo 7 as a probe with nuclear extract derived from normal spleen B cells stimulated with LPS (10 μg/ml).

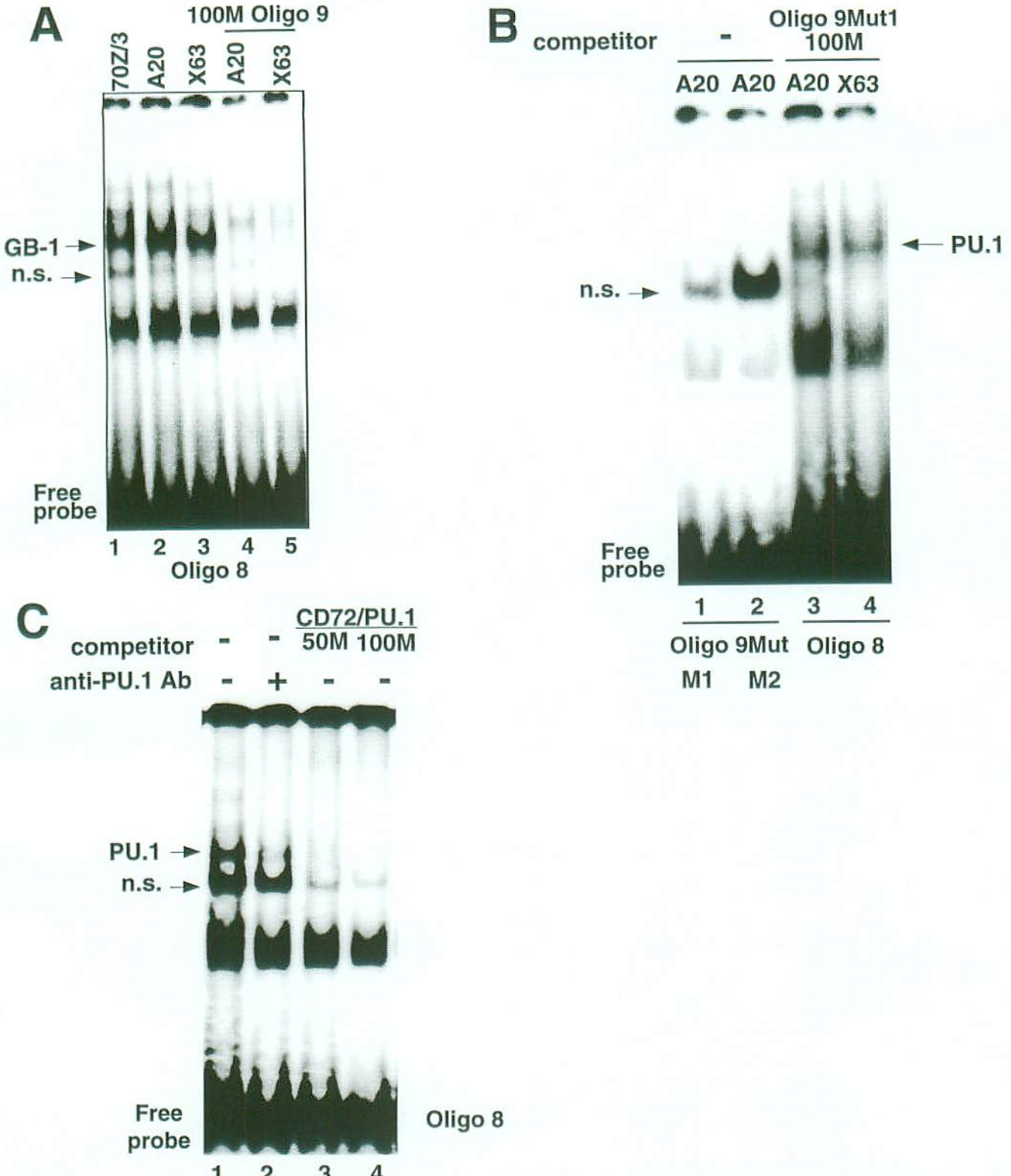


Figure 6. DNA-protein interaction on *ganp* promoter. ***A***, The DNA-fragment from –134 to –107 bp ofthe *ganp* promoter region is bound by a B cell-specific protein complex. EMSAs were performed as described under "Experimental Procedures". A double strand oligonucleotide (Oligo 8) was radiolabeled and incubated with nuclear protein extracts from the indicated cell lines. In *lanes* 4 and 5, 100 molar excess of an unlabeled nucleotide (Oligo 9) was used as a specific competitor. *GB*-1 denotes the specific DNA-protein complex detected; n.s., a non-specific band. ***B***, The PU.1-binding site within the sequence –134 to –107 bp is required for the formation of the GB-1 complex. Oligo 9, containing the 15- nucleotide region with the PU.1 site from *ganp* promoter, was mutated by replacing the PU.1 site alone (*M1*) or the PU.1 site and the neighboring 5'-GGA-3' sequence (*M2*), and they were radiolabeled for *lanes* 1 and 2. Unlabeled Oligo 9Mut *(M1)* was used as a specific competitor in *lanes* 3 and 4. ***C***, Anti-PU.1 Ab specifically inhibits binding of PU.1 to the *ganp* promoter. The labeled Oligo 8 was incubated with nuclear extract from A20 cells. Anti-PU.1 Ab (4µl) was added in *lane* 2. The unlabeled DNA fragment, CD72/PU.1, which contains a known PU.1 site from the CD72 promoter, was used in 50 and 100 molar excesses in *lanes* 3 and 4 as a specific competitor.

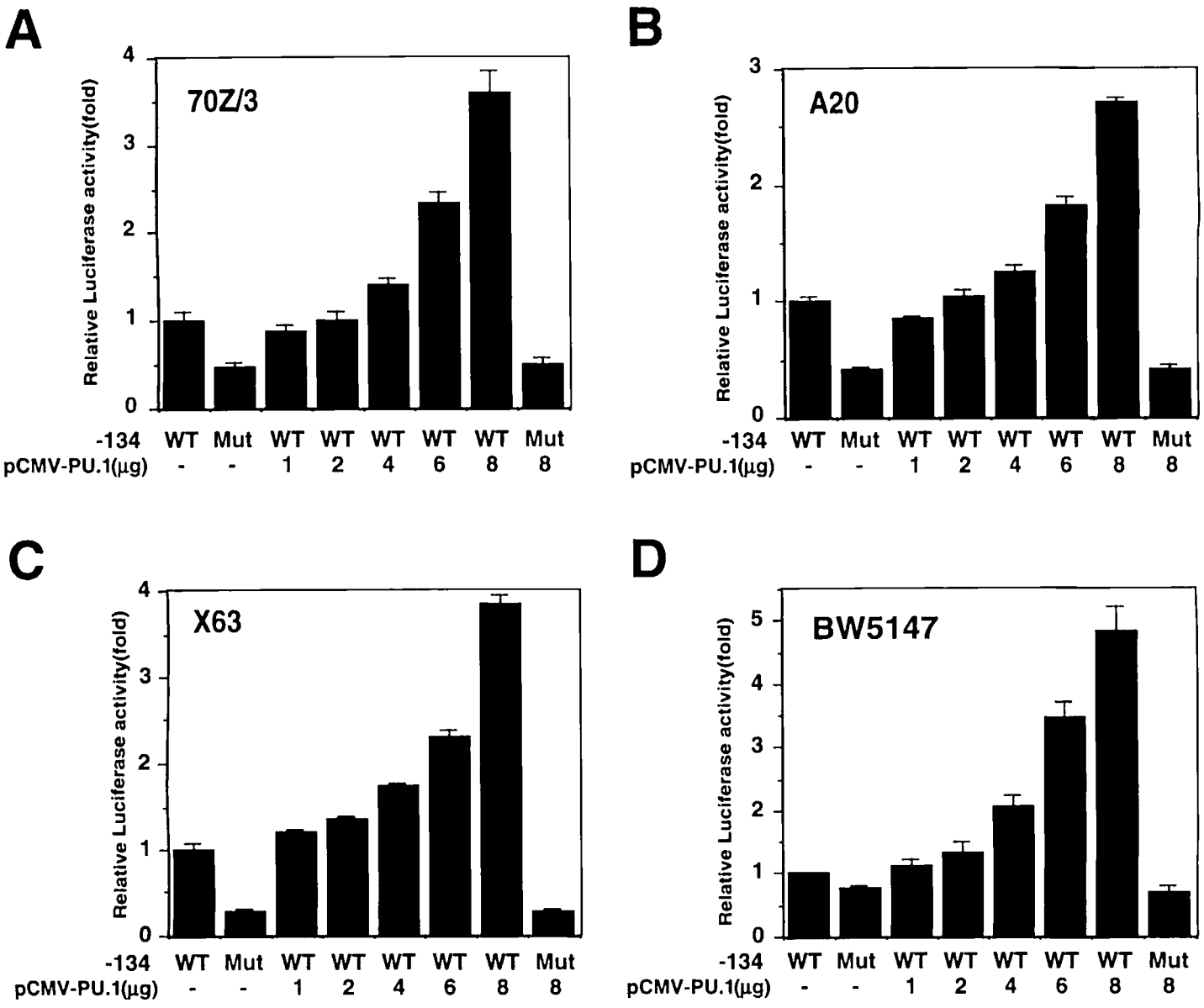


Figure 7. Reporter gene activity of *ganp* promoter PU.1 element of the –134-bp construct. Overexpression of PU.1 increases the promoter activity of the constuct –134 bp with which the regulatory activity of the PU.1 site was measured. The wild-type *(WT)* or the mutant *(Mut)* reporter construct of the –134-bp region was co-transfected with the full length *PU.1* cDNA in the expression vector pCMV. Luciferase analysis was performed as described under "Experimental Procedures", and the results were expressed as fold activity relative to the wild-type control construct (assigned a value of 1). Each histogram represents the value of luciferase activity (±S.D.) of three transfection experiments. In the case of the control, an equivalent amount of DNA from the relevant empty vector was co-transfected with the reporter construct. The mutation introduced into the PU.1 site changed the sequence 5'-GGAA-3' to 5'-AATT-3'.

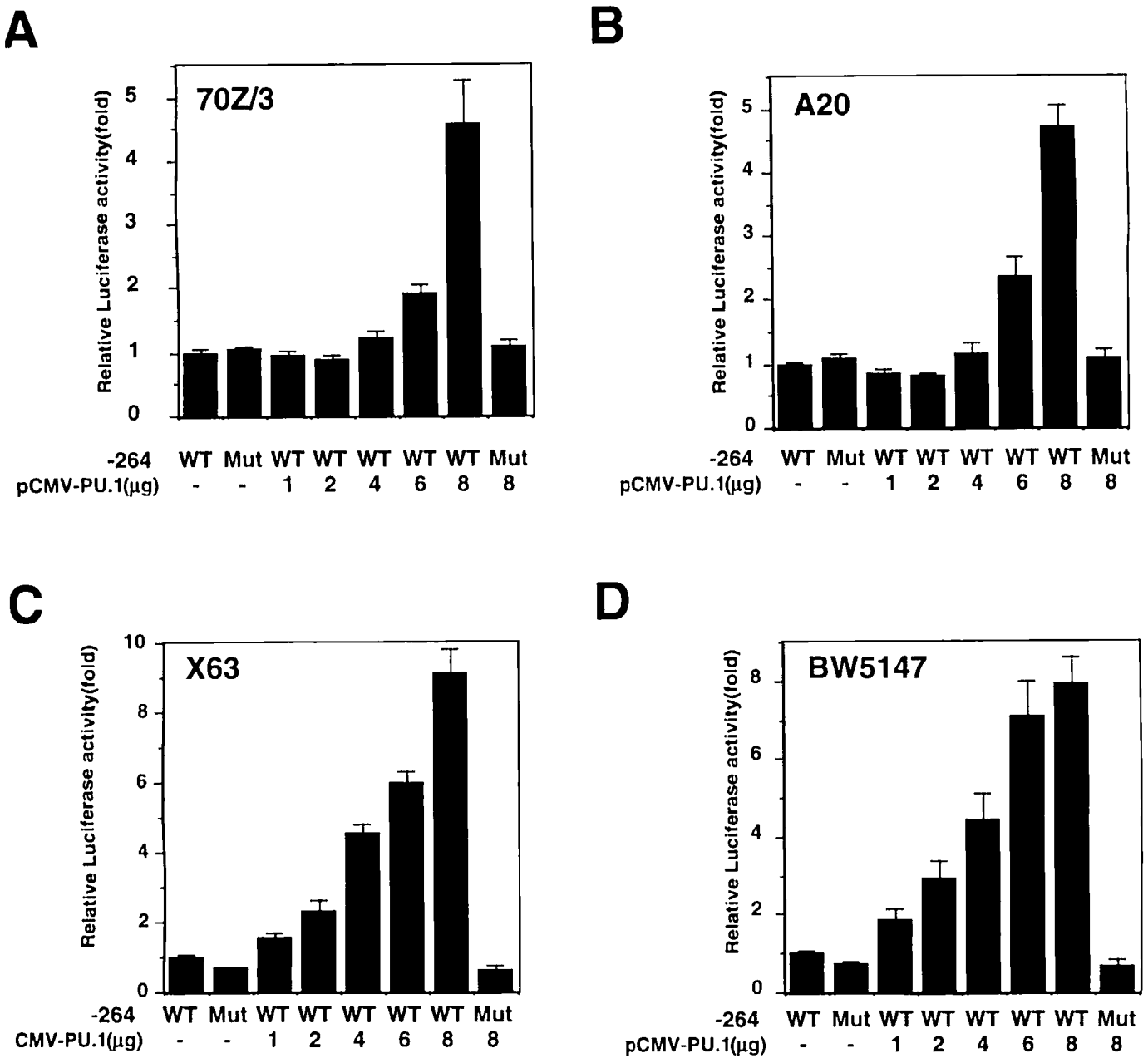
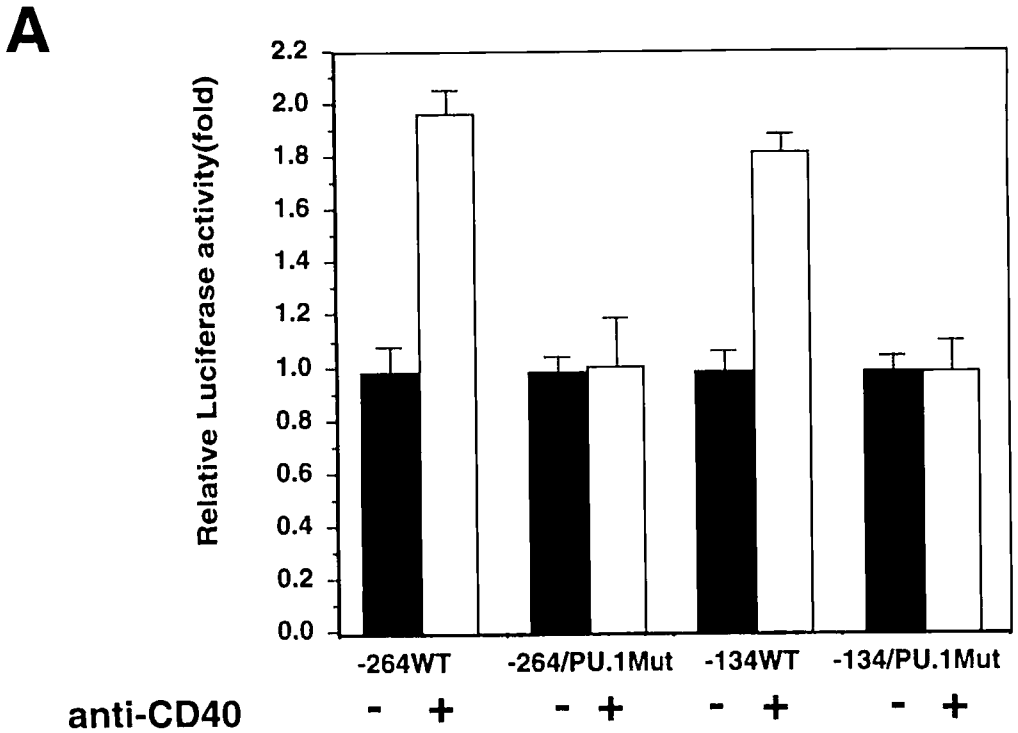


Figure 8. Reporter gene activity of *ganp* promoter PU.1 element with the promoter construct –264 bp. Point mutations introduced into the PU.1 binding site within the promoter construct –264 bp and the co-transfection was carried out as described for Fig. 4. The induction of the promoter activity was measured after introduction of PU.1 as relative luciferase activity in 70Z/3 (*A*), A20 (*B*), X63 (*C*), and BW5147 (*D*).



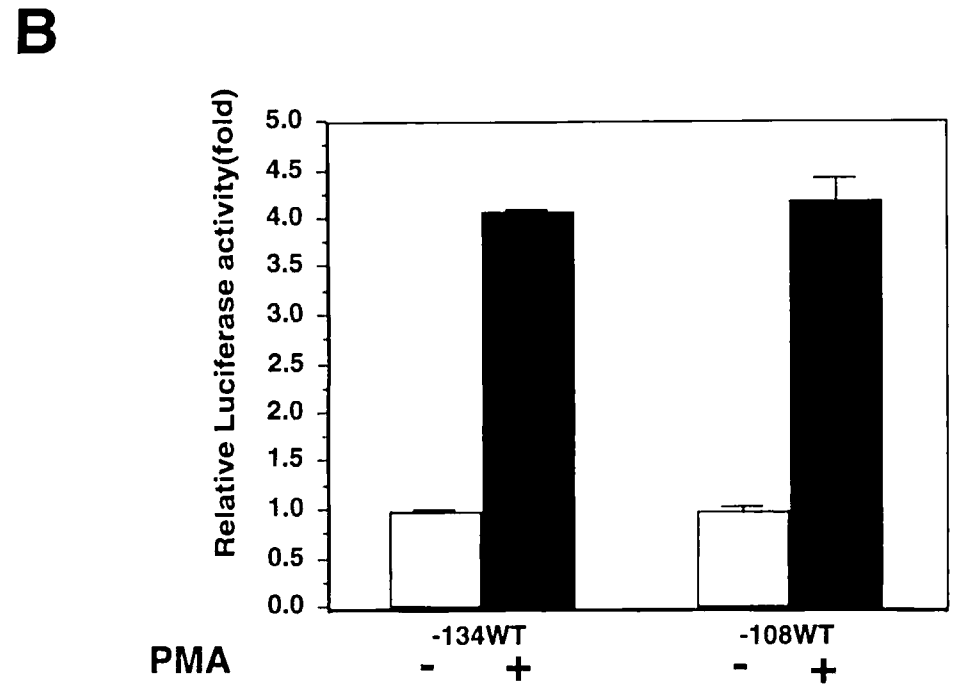


Figure 9. **B cell stimulation induces up-regulation of the *ganp* promoter activity.** ***A***, mature B cells (A20) were transfected with either the wild-type or the PU.1-mutant luciferase reporter construct of the –134-bp and –264-bp *ganp* promoter region and then stimulated for 48 h with anti-CD40 (10μg/ml) as indicated. ***B***, A20 cells were transfected with the promoter construct -134 or -108-bp and then stimulated with for 48 h with PMA (10 ng/ml). Results are presented as a fold increase in luciferase activity relative to a control transfection without stimulation (assigned a value of 1). Values (mean ± S.D.) from two independent transfection experiments are shown. The experiment was repeated at least twice in triplicates.

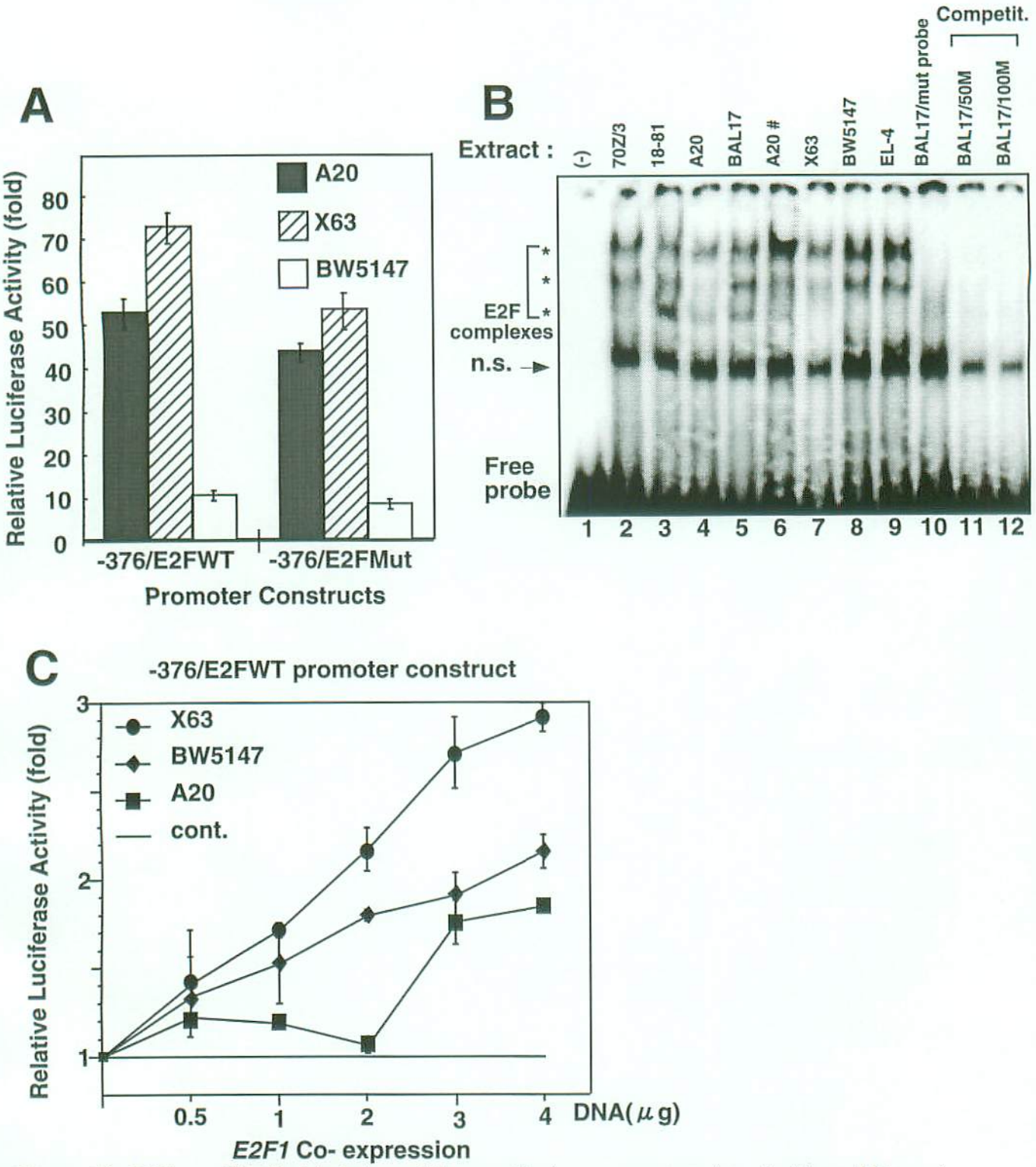


Figure 10. E2F specifically binds to and transactivates *ganp* promoter. ***A***, The wild-type (-376/E2FWT) construct and the mutant (-376/E2F/Mut) construct in which the sequence 5'-TTTGGGG-3' was changed to 5'-CGATGTA-3' were transfected into the indicated cells. Transfection and luciferase analysis were performed as described under "Experimental Procedures". The relative luciferase activity is expressed as a fold increase above the background conferred by the promoterless/enhancerless control plasmid. Each histogram represents the mean (±S.D.) of two independent experiments. The experiment was repeated three times. ***B***, EMSA using an oligonucleotide probe containing the E2F binding site of the *ganp* promoter. Oligo E2F (see Fig. 3A) was radiolabeled and incubated with nuclear extract prepared from the indicated cells. In *lane* 10, the probe contained the same point mutation indicated in *A*. 50 and 100 times molar excess of the unlabeled oligo E2F/*wt* were used as a specific competitor in *lanes* 11 and 12. The specific E2F complexes and the cold probe are indicated to the left of the photograph.n.s.,non-specific band. ***C***, An expression vector encoding the full length *E2F1* cDNA was cotransfected with the wild-type -376/*E2F/wt* reporter construct into the indicated cells. In case of control, an equivalent amount of DNA of the relevant empty plasmid was co-transfected with the reporter construct. The luciferase activity is expressed as a fold increase above the background conferred by the control (assigned a value of 1).

4. Discussion

GANP is a new member of RNA/DNA-primase that associates with the DNA-helicase complex of MCMs, and interestingly both the expression and the activity of DNA-primase are induced in Ag-driven B cells in GCs (Kuwahara *et al.*, 2001). The GANP may function in various ways; GANP may be involved in DNA replication of not only GC-B cells but also of various other cells, especially in rapid proliferation.

As with many lymphoid-specific genes, the mouse *ganp* promoter lacks an obvious TATAA box and initiates transcription at multiple sites. The initiator element, which is present in promoters with or without TATAA box, is believed to be necessary for positioning the transcription initiation complex in promoters lacking the TATAA box (Weis and Reinberg, 1992). Interestingly, the sequence element 5'-CACA(N)$_5$GAGNC-3' encompassing nucleotides -174 to -161 of the *ganp* promoter shows a 8 of 9-bp identity among 14 nucleotides at its 5' and 3' ends with the TdT initiator sequence 5'-CTCA(N)$_5$GAGNC-3' (Winter *et al.*, 2000).

Analysis of transcription activity in the 5' putative *ganp* promoter region of –737-bp demonstrates that the region serves for the B lineage-associated and developmental stage-dependent regulation in cell lines maintained *in vitro*. Transient transfection analysis indicates that the *ganp* promoter is much more active in B cells than in T cells. Importantly, the *ganp* promoter is increasingly active as the B cell maturation proceeds, and this is consistent with a previous study showing that *ganp* expression is predominant at a later stage(s) of B cell development (Kuwahara *et al.*, 2000 and 2001). The *ganp* promoter is less active during the pre-B cell stage, but the activity increases along with the maturation of B cells to sIgM-positive B cells and is at its highest in terminally differentiated plasma cells. Such an increase in the promoter activity through B cell differentiation is not caused by variations among the individual cell lines analyzed, because transfection of other cell lines representing similar stages

and lineages displayed equivalent profiles of *ganp* promoter activity (data not shown).

We determined the element recognized by PU.1 as one of the regulatory motifs involved in the regulation of *ganp* gene expression. PU.1 is an Ets family transcription factor specifically expressed by erythroid, myeloid, and B cells (Klemsz *et al.*, 1990; McKercher *et al.*, 1996; Fisher *et al.*, 1998; Henkel *et al.*, 1999; Nerlov and Graf, 1998), and may be crucial for the tissue-specific regulation of Ig κ-chain gene rearrangement (Hayashi *et al.*, 1997; Hiramatsu *et al.*, 1995). Gene-targeting experiments of the mouse *PU.1* locus demonstrate the necessity of PU.1 for the development of B cells and terminal differentiation of macrophage lineage (Scott *et al.*, 1994). PU.1 has been reported to interact with important regulatory regions in the promoters of many B cell-specific genes including the J chain promoter (Shin and Koshland, 1993), Ig κ 3' enhancer (Pongubala *et al.*, 1992), heavy chain *Eμ* enhancer (Linderson *et al.*, 2001), *Btk* promoter (Himmelmann *et al.*, 1996), *CD18* (Rosmarin *et al.*, 1995) and *IL-1β* (Kominato *et al.*, 1995) promoters. All of these genes are required for the proper differentiation of B cells. In a more striking example for the role of PU.1 in the molecular function of B cells, it was found that PU.1 in cooperation with an adjacent binding motif is responsible for directing somatic hypermutations in the V region of Ig κ gene. These studies suggest that PU.1 plays an important a role in the regulation of several genes necessary for molecular processes required for B cell proliferation and differentiation.

The PU.1 consensus site at −126-bp of the *ganp* gene promoter region is recognized by the component including PU.1 in B lineage cells. This was confirmed by cold inhibition (shown on EMSAs), and the mutation at the PU.1 consensus site abolished the binding completely. The anti-PU.1 Ab inhibited the formation of the DNA-protein complex (with only a low level of the complex remaining), which is presumably recognized by related transcription factors such as Spi-B (Su *et al.*, 1997). The reporter assay clearly demonstrates the positive effect of PU.1 on the PU.1 site (-

126-bp), whose mutation abolished the up-regulation by exogenous *PU.1* cDNA co-transfection. In the shorter construct, the mutation clearly reduced the spontaneous promoter activity in B cell lines; however, the longer *PU.1-mutated* construct (-264-bp) with other putative consensus sites did not show such a decrease. The longer construct with the mutation did not respond at all to the exogenous *PU.1* cDNA.

The mechanism of how the longer *PU.1-mutated* construct did not show a decrease in B cells remains to be determined. It seems that the transcriptional activity of PU.1 might be suppressed by the distal element(s) or the molecules expressed at pre-B and B cells. This notion my be consistent with the gap decrease of the reporter activity only in A20 after deleting the promoter element from –264 to –168-bp in which the BSAP consensus site is found (Fig. 3A). A BSAP-like protein complex (GB-X) was detected with oligo 7 only in 70Z/3 and A20 cells but not in X63 or BW5147 (Fig. 5). BSAP (Pax5) is a bifunctional transcriptional factor capable of repressing and activating transcription (Maitara and Atchison, 2000). Genes activated by BSAP involve molecules expressed during early B cell differentiation (Hagman *et al.*, 1991; Kozmik *et al.*, 1992), but the genes repressed are expressed mostly at the later stage of maturation as the heavy chain enhancer element, the J chain gene, and the *PD1* gene (Singh and Birshtein, 1993; Rinkenberger *et al.*, 1996; Nutt *et al.*, 1998). BSAP represses PU.1 activity through binding to the transactivation domain, although BSAP does not affect PU.1 binding activity to the PU.1 site (Maitara and Atchison, 2000). Pre-B cells show the lower reporter activity in comparison with A20 with all of the truncated constructs, suggesting that an additional proximal region(s) is involved in repressing the activity of *ganp* gene expression at the pre-B cell stage. Because GANP is up-regulated in Ag-driven B cells *in vivo*, an activation-inducible region is particularly important. Signals via CD40 and BCR are known to synergize in B cell activation with induction of the nuclear transcription factor NF-κB/Rel (Ubanek *et al.*, 1994). There are no apparent binding sites for NF-κB in the *ganp* promoter region

analysed here, and the inactivation of IκB did not show any effect on reporter gene activity (data not shown). On the contrary, CD40 signaling showed a positive effect on *ganp* promoter activity that is mediated through the PU.1 site, suggesting that PU.1 might be involved in the up-regulation of endogenous GANP in GC-B cells stimulated with Ag and CD40/CD40L interaction. The effect of anti-CD40 stimulation on A20 cells is not strong in comparison with the exogenous introduction of *PU.1* cDNA, but the mutation at the PU.1 site abolished the enhancement induced by stimulation with anti-CD40 mAb indicating that PU.1 site in *ganp* promoter is probably a direct target of CD40 signaling pathway. Some members of the Ets family of transcription factors have also been implicated in coordinating responses required for proliferation, differentiation and survival. Signaling pathways such as the MAP kinases, Erk1/2, JNK, and PI3 kinases activated by growth factors or cellular stresses, converge on some Ets family of transcription factors, controlling their activity, protein partnerships, and specification of downstream target genes (Yordy and Muise-Helmericks, 2000). A comprehensive view of the possible signaling pathways that may link CD40 signal and PU.1 induction of *ganp* expression is shown in Fig. 11. The results presented here are based on the reporter gene assay using cell lines maintained *in vitro*. Normal naive B cells in the quiescent cell cycle stage do not express GANP protein at a detectable level, but GANP expression is up-regulated by immunization with T-dependent antigen *in vivo* and by stimulation with anti-CD40 mAb *in vitro*. There might be difference in the states of nuclear transcription factors between the resting normal B cells and the proliferating B cell lines, especially regarding the observation that GANP expression is controlled by the cell cycle-associated transcription factors such as the E2F. E2F1 directly activates *ganp* transcription in plasma cells and T cells, but the kinetics of the response differed in mature B cells. E2F expression is mostly at a lower level in the resting cells (Iciek *et al.*, 1997; Adams and Kaelin, 1996; Lindeman *et al.*, 1997), and is probably similar in centrocytes whose cell proliferation is arrested in GCs.

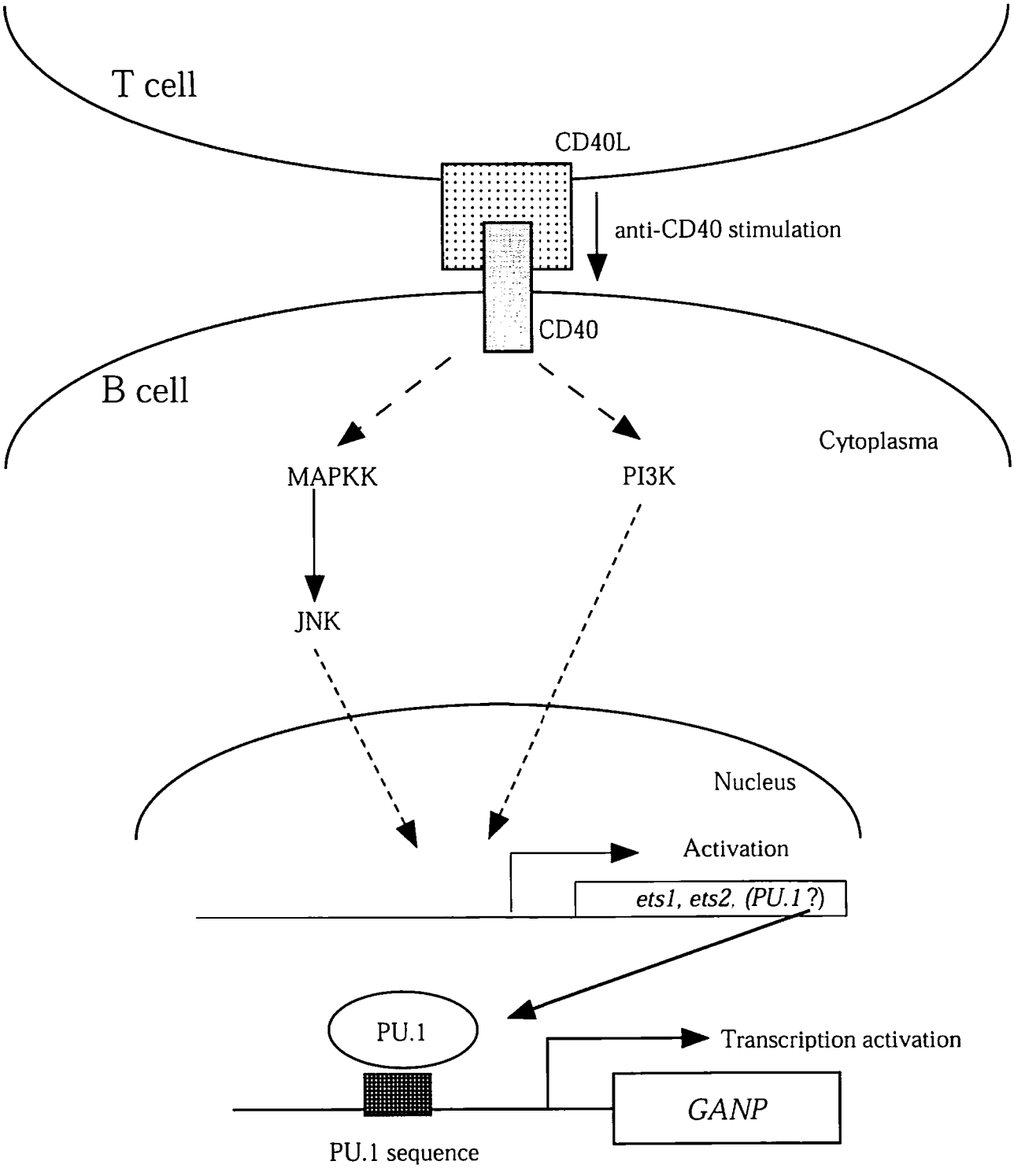


Figure 11: A schematic representation of signaling pathways that may relay signals from CD40 to PU.1, leading to activation of *ganp* expression.

To accord with the proliferation rate, it is assumed that the MCM-complex required for DNA replication should be up-regulated. The differential expression of MCM3 and GANP during maturation of GC-B cells would imply a unique role of GANP expression, which might be necessary after DNA replication. In addition, expression of genes involved in the cell cycle control as well as DNA replication is closely correlated with the proliferation state of the cell and, increases dramatically at late G1 phase in response to growth stimulation (Matsukage *et al.*, 1986; Baserga, 1991). Many of these genes including the proliferating cell nuclear antigen (PCNA), MCM3, DHFR, Cdc6, DNA polymerase α, and cyclin A contain E2F consensus sequence elements in their promoters, or are regulated by E2F activity. The E2F family of transcription factors plays an important role in the regulation of gene expression at the G1/S phase transition of the cell cycle (Dyson, 1998). These E2F target genes display cell cycle-stage-specific transcriptional regulation (Wells *et al.*, 2000). With the exception of c-myb, the majority of these genes are activated at or near the G1/S phase (Campanero *et al.*, 1999). E2F transcription factors play a role in the regulation of cell cycle by restricting the expression of these genes to the point of the cell cycle where their respective products function. While in quiescent or differentiated cells, the retinoblastoma suceptbility gene product pRb and its related family members are hypophosphorylated (active) and most of E2F proteins are bound, in cells stimulated to proliferate, pRb proteins become phosphorylated or inactive by cyclin-dependent kinases and this results in the release of active E2F proteins and hence transcription activation (Campanero *et al.*, 1999). Thus, transcriptional activation of many E2F-regulated genes correlates with the phosphorylation of pRb proteins at the G1/S phase boundary (Campanero *et al.*, 1999), and the cell cycle regulator E2F and its related proteins are determinants for DNA replication and growth control.

The data presented here showed a positive role of the E2F on *ganp* expression in most of cells, but suggested that E2F regulates *ganp* expression differently in mature B

cell lines that might be similar to GC-B cells. A recent report showed that the *in vitro* stimulation with anti-IgM and anti-CD40 Abs efficiently induced the expression of E2Fs from the very low level in the resting B cells (Lam *et al.*, 1999). While the magnitude of E2F effect is not much in the reporter assay, it may represent the stage dependent-regulation of GANP expression during normal B cell maturation from centroblasts to centrocytes. The E2F binding site has been suggested to alternate between a positive and negative regulatory element with the phosphorylation and dephosphorylation state of Rb protein (Weintraub *et al.*, 1992). It would be important to know the levels, phosphorylation status, protein partnership, activity of E2F pocket proteins and whether the E2F4/p130 complex could be replaced by E2F1 and E2F4 with p107 or pRb, resulting in the alteration of their activity on *ganp* transcription with the cell cycle progression in Ag-driven B cells.

The *ganp* promoter is regulated throughout B cell development and is the target of cell signaling events via PU.1 cis-acting regulatory element(s). This information would also be helpful to characterize the stage-dependent regulatory mechanism that might regulate the expression of other functional molecules required for the activation of Ag-specific B cells in differentiating into Ab-secreting plasma cells.

5. Conclusions

This study isolated the 737-bp *ganp* promoter region, and determined the initiation sites of the *ganp* transcripts. The results obtained by EMSA and luciferase reporter assay demonstrated that the *ganp* promoter region is responsible for the B lineage-associated and stage-dependent transcription regulation, as being less active during the pre-B cell, more active during the mature B cell, and most active during the terminally differentiated plasma cell stage. The PU.1 sequence element of the *ganp* promoter is largely responsible for the selective induction of *ganp* expression in B cells. PU.1 mediates this function through possible interaction with other regulatory sequences, most likely the BSAP sequence element at –235-bp. As the cell cycle-associated control, the E2F sequence element at –357-bp might play a role in the regulation of *ganp* expression during proliferation and cell cycle transition.

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