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Regulation of DNA metabolic enzymes upon induction of preB cell development and V(D)J recombination: up-regulation of DNA polymerase δ

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ABSTRACT

Withdrawal of interleukin-7 from cultured murine preB lymphocytes induces cell differentiation including V(D)J immunoglobulin gene rearrangements and cell cycle arrest. Advanced steps of the V(D)J recombination reaction involve processing of coding ends by several largely unidentified DNA metabolic enzymes. We have analyzed expression and activity of DNA polymerases α, β, δ and ε, proliferating cell nuclear antigen (PCNA), topoisomerases I and II, terminal deoxynucleotidyl transferase (TdT) and DNA ligases I, III and IV upon induction of preB cell differentiation. Despite the immediate arrest of cell proliferation, DNA polymerase δ protein levels remained unchanged for ~2 days and its activity was up-regulated several-fold, while PCNA was continuously present. Activity of DNA polymerases α, β and ε decreased. Expression and activity of DNA ligase I were drastically reduced, while those of DNA ligases III and IV remained virtually constant. No changes in DNA topoisomerases I or II expression and activity occurred and TdT expression was moderately increased early after induction. Our results render DNA polymerase δ a likely candidate acting in DNA synthesis related to V(D)J recombination in lymphocytes.

INTRODUCTION

The assembly of variable region exons of the antigen receptor loci [V(D)J rearrangement] is one of two DNA recombination processes which are the key to the generation of a highly diverse immune repertoire (1; reviewed in 2–5). The V(D)J recombination machinery (reviewed in 6–11), a complex set of enzymes and accessory proteins, is directed to the V, D and J gene elements through recombination signal sequences (RSS) adjacent to each exon. Initiation of V(D)J recombination, including cleavage at the RSS and formation of hairpin coding ends, has recently been shown to be catalyzed by purified RAG-1 and RAG-2 proteins (12). From genetic complementation studies with rodent cell mutants, DNA-dependent protein kinase (DNA-PK) holoenzyme with its three subunits, DNA-PK_{cs}, Ku70 and Ku86, was found to be involved in the early steps of V(D)J recombination (for reviews see 13–16). A similar approach led to the identification of the *XRCC4* gene (17), mutants of which are defective in V(D)J recombination. At later steps, DNA fill-in synthesis of open coding ends may generate blunt ends and lead to the insertion of templated nucleotides (P-nucleotides). Subsequent modification by non-templated N-nucleotide addition through the action of terminal deoxynucleotidyl transferase (TdT; 18) and nucleolytic trimming of the DNA ends coupled with DNA repair synthesis often occurs before the ends are ligated to form the coding join.

At least five different DNA polymerases (pols), called α, β, γ, δ and ε, are known to exist in higher eukaryotic cells (reviewed in 19,20). Pol α is responsible for initiation of DNA replication at the origin of replication (21). Pol β might act preferentially in base excision repair (22). Pol γ is the replicase of the mitochondrial genome. Pol δ is involved in nuclear DNA replication at the leading strand and possibly also at the lagging strand of the replication fork (21). On many DNA substrates, pol δ requires the two auxiliary proteins proliferating cell nuclear antigen (PCNA) and replication factor C (RF-C) for processive synthesis (reviewed in 23). A function for pol ε has been proposed in nuclear DNA repair, but it appears to be important for nuclear DNA replication as well, possibly as a second lagging strand polymerase (24), since its gene is essential in yeast. In nucleotide excision repair, pol ε relies, as does pol δ, on PCNA and RF-C (25) and the enzyme has been identified in a protein complex that repairs DNA double-strand breaks and deletions by recombination (26). Finally, pol ε appears to have a role in check-point control of the cell cycle (27).

Four DNA ligases have been discovered in mammalian cells (for reviews see 28,29). DNA ligase I is best known as the predominant and replicative, Okazaki fragment joining ligase in many tissues. Less is known about the functions of DNA ligases II, III and IV. DNA ligase II was found to be the most abundant DNA ligase in mammalian liver (30). DNA ligase III associates with XRCC-1, a DNA repair protein (31), and co-purifies with the recombination protein complex RC-1 (26). Furthermore, it is the major high molecular weight DNA joining activity in SV40-transformed human fibroblasts (32). A role for DNA ligase III in meiotic recombination in mammalian cells has also been postulated (33). Amino acid sequence data suggest that DNA

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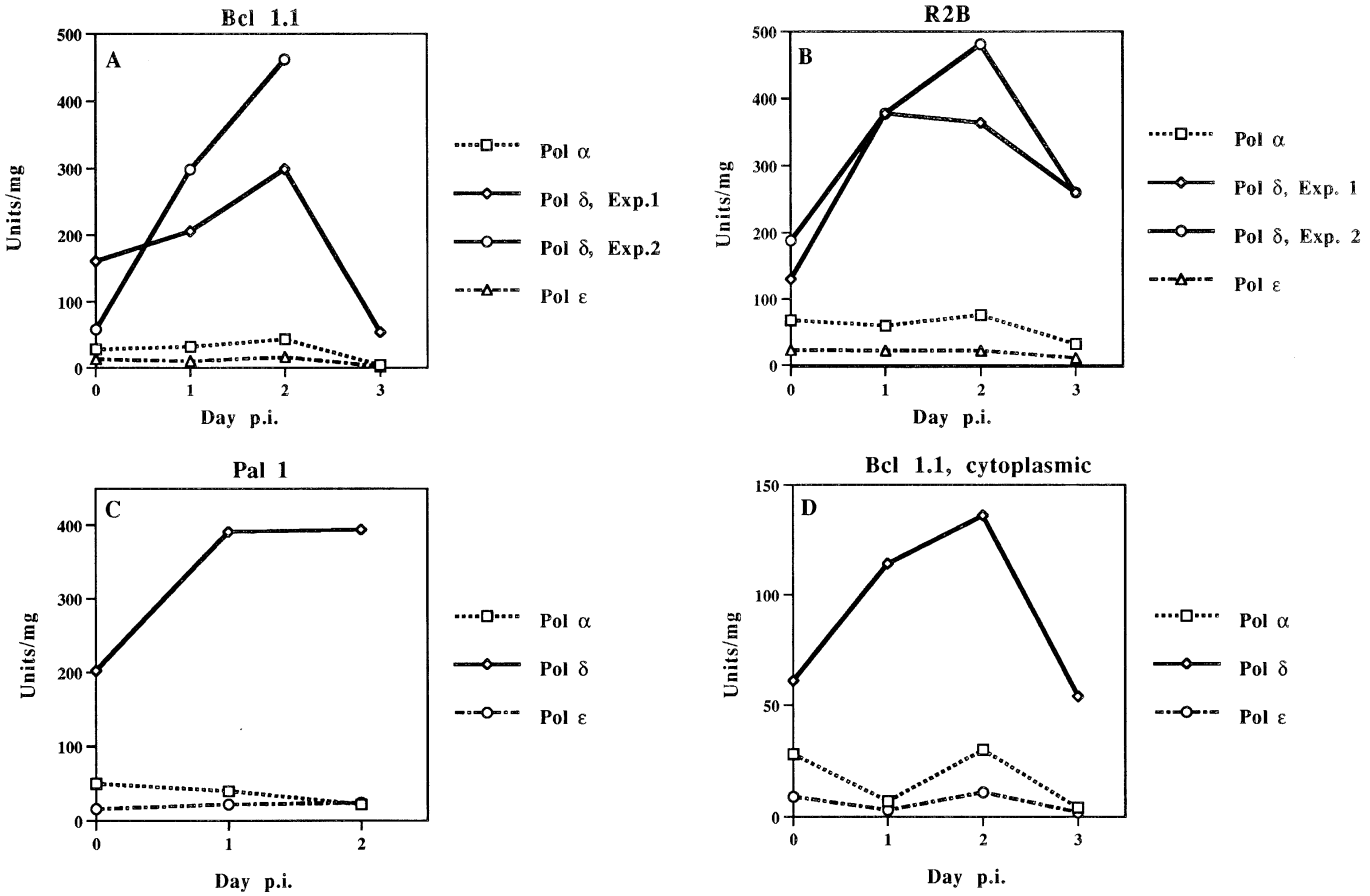

Figure 1. DNA polymerase activities before and after induction of preB cell differentiation. Nuclear extracts of Bcl1.1 (**A**), R2B (**B**) and Pal 1 (**C**) preB cells and of Bcl (**D**) were assayed for pol α, δ and ε as described in Materials and Methods. p.i., post-induction.

ligase II might be encoded by the same gene as DNA ligase III (33). The more distantly related DNA ligase IV has been discovered only recently and, so far, nothing is known about its *in vivo* function (34). The cDNAs encoding the mammalian DNA ligases I, III and IV have been isolated and sequenced.

The DNA polymerase and ligase involved in processing of V(D)J recombination intermediates have not been specified. It is also unknown how these enzymes and their accessory factors, as well as other DNA metabolic activities like TdT or topoisomerases, are regulated during development of lymphocytes and upon cell cycle arrest. A tissue culture system has been established for preB lymphocytes which mimics many aspects of development of early B cells in the bone marrow (35,36). In this system, preB cells proliferate on stromal cells in the presence of interleukin-7 (IL-7). Only heavy chain D to J rearrangements have been completed prior to this proliferative stage. Upon withdrawal of the cytokine, proliferation ceases and differentiation to surface immunoglobulin-positive (sIg^+) B cells is induced. The non- replicating lymphocytes are no longer hydroxyurea sensitive. Since most of the differentiating B lymphocytes normally enter apoptosis and would not be available for analysis, preB cell cultures from mice carrying the anti-apoptotic *bcl*-2 transgene (37) were established. These *bcl*-2 preB cells match wild-type preB cells in all properties examined, except apoptotic death. Hallmarks of their development are up-regulation of expression of the recombination activator genes 1 and 2 (*RAG*-1 and *RAG*-2; 38) and rearrangements of their Ig heavy chain loci. The cells continue *RAG* expression, rearrange their light chain gene loci (36,39,40) and become sIg^+ B cells within 2–3 days. This B lymphocyte differentiation system lends itself to the molecular analysis of V(D)J rearrangement, B cell development, cell cycle arrest and the DNA metabolic enzymes associated with these processes.

MATERIALS AND METHODS

Cell lines

Stromal cell/IL-7-dependent preB cell lines were obtained from day 16 fetal liver or 4–8-week-old mouse bone marrow and cultured as described (35,36). Three different preB cell lines were used in this study: Bcl1.1, a wild-type *bcl*-2 transgenic mouse derived line; Pal 1, a wild-type non *bcl*-2 transgenic line; R2B, a *bcl*-2 transgenic line derived from a *RAG*-$2^{-/-}$ mouse (41).

Immunoblotting and immunoprecipitation

To analyze PCNA, DNA topoisomerases I and II, TdT and DNA ligases, cytoplasmic and nuclear extract fractions were prepared from 1×10^6–5×10^7 cells as described (26,40). Cytoplasmic fractions contained 1–5 mg/ml nuclear fraction, 0.2–2 mg/ml protein. Total cell lysates were prepared according to Lin and Desiderio (42). Western blotting was performed according to standard protocols and visualization of signals achieved with the

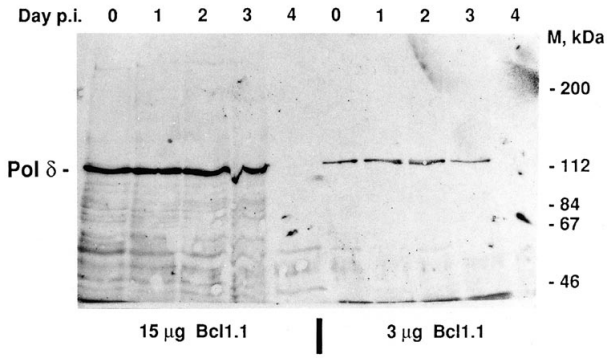

Figure 2. Immunoblot analysis of DNA pol δ expression before and after induction of differentiation. Total cell lysates from wild-type Bcl1.1 preB cells (15 and 3 µg protein as indicated) were separated by 7.5% SDS–PAGE and probed with a polyclonal antibody specific for the 125 kDa subunit of pol δ (43). Positions of the 125 kDa pol δ and of size markers are indicated. p.i., post-induction.

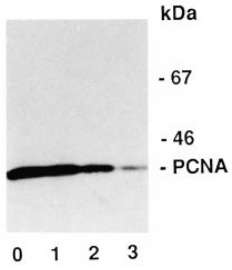

Figure 3. Immunoblot analysis of PCNA expression before and after induction of differentiation. 10 µg total cell lysate protein were separated by 7.5% SDS–PAGE and probed with monoclonal antibody PC10. The position of 36 kDa PCNA is indicated. Numbers at the bottom refer to days post-induction.

ECL system (Amersham Ltd). For immunoprecipitation of DNA ligase I, ~3–5 × 10^7 cells were metabolically labeled for 5 h with [^{35}S]methionine, harvested and nuclear and cytoplasmic extracts prepared as outlined above. Antibody (rabbit polyclonal sera TL5) binding was carried out with 10 µg extract protein (~1 × 10^7 c.p.m.) in the presence of 0.05% Triton X-100, the antibody–antigen complex being precipitated by protein A–Sepharose beads (Pharmacia) and, after several wash steps, loaded on a 7.5% SDS-polyacrylamide gel. The gel was dried and exposed for autoradiography.

DNA polymerase assays

Cells were thawed on ice for 30 min, resuspended in 3 vol extraction buffer [10 mM Tris–HCl, pH 7.5, 10% (v/v) glycerol, 0.5 mM EDTA, 10 mM KCl, 0.25 M sucrose, 10 mM benzamidine, 5 µg/ml pepstatin, 1 µg/ml leupeptin, 1 mM PMSF and 1 mM DTT] and sonicated twice for 10 s in a Branson Sonifier (setting 5). After 10 min on ice the extract was centrifuged at 12 000 *g* for 30 min. The supernatant was designated cytoplasmic extract. The pellet was resuspended in the same volume of extraction buffer containing 0.4 M NaCl, left for 10 min on ice and centrifuged as above, yielding the nuclear extract.

PCNA was removed from the extracts as follows. Aliquots of 50 µl cytoplasmic or nuclear extract were mixed in an Eppendorf tube with 20 µl phosphocellulose equilibrated in equilibration buffer [40 mM Tris–HCl, pH 7.8, 50 mM KCl, 20% (v/v) glycerol, 0.5 mM EDTA, 0.2 mM PMSF and 1 mM DTT] and the protein adsorbed for 60 min by rotating the tubes in the cold. Then the phosphocellulose was washed three times with 200 µl equilibration buffer and the polymerases eluted with 40 µl equilibration buffer containing 0.4 M NaCl. This procedure yields two fractions containing no PCNA but pol α, δ and ε (44).

The DNA polymerase assays for pol α, δ and ε were based on our earlier studies (44) with PCNA-free extract. Activated DNA gave an estimation about the total level of DNA polymerases and was used as an indication of successful extraction. Poly(dA)·oligo(dT) can be used in combination with PCNA and the pol α neutralizing antibody SJK 132-20 to specifically determine pol α, δ and ε. The rationale behind the assays is: (i) poly(dA)· oligo(dT) alone gives pol α and ε (pol δ is inactive without PCNA); (ii) poly(dA)·oligo(dT), 100 ng purified PCNA and 3 µg SJK 132-20 give pol δ and pol ε; (iii) poly(dA)·oligo(dT) and 3 µg SJK 132-20 give pol ε; (iv) (i) minus (iii) gives pol α; (v) (ii) minus (iii) gives pol δ.

Assays with activated DNA or poly(dA)·oligo(dT) were performed as described (44).

Extraction and determination of DNA polymerase β were performed according to Hübscher *et al.* (45). One unit is defined as the incorporation of 1 nmol dTTP in 1 h at 37°C and activity was calculated as U/mg protein.

DNA ligase assays

After thawing aliquots of Bcl1.1 cells [2–3 × 10^8 cells, day 0 and day 2 post induction (p.i.)] on ice, crude nuclear extracts were prepared according to the method described (46). To separate the two 100 kDa DNA ligases III and IV from each other, the crude nuclear extracts (0.3– 0.7 mg protein) were fractionated by ion exchange chromatography (47). DNA ligases in the individual fractions were then identified by Western blotting and activity assays.

For Western blots samples (50 µl) of the nuclear extract fractions, alongside 5 µg HeLa cell nuclear extract, were separated by 8% SDS–PAGE (three identical gels each for fractions from day 0 and day 2 p.i. cells) and electrotransferred (1 h, 350 mA in 10% methanol, 25 mM Tris, 192 mM glycine) to Protran-Nitrocellulose (Schleicher & Schuell), followed by the standard protocol supplied with the ECL detection system. Ligase I detection was achieved by incubation with a rabbit antiserum raised against homogeneous bovine DNA ligase I (48) at a dilution of 1:250 for 1 h at 20°C. Ligases III and IV were detected with a rabbit antiserum against recombinant human DNA ligase III and an affinity purified rabbit antiserum against a synthetic peptide of human DNA ligase IV respectively (47).

DNA ligation was assayed with the double-stranded polynucleotide substrates [5′-^{32}P]oligo(dT_{16})·poly(dA) and [5′-^{32}P]oligo(dT_{16})·poly(rA). The substrates were made as described previously (49). Samples (4 µl) of the nuclear extract fractions were incubated in reaction mixtures (30 µl) containing 60 mM Tris–HCl, pH 8.0, 10 mM $MgCl_2$, 50 µg/ml BSA, 5 mM DTT, 1 mM ATP and 0.1 µg substrate DNA (20 000–50 000 c.p.m.) at 37°C. Aliquots of 5 µl were removed after 15 min and the reactions were stopped by adding 20 µl formamide (90%) dye, heating at 95°C for 5 min and immediately cooling on ice. ^{32}P-Labeled oligo(dT) multimers were resolved by electrophoresis through denaturing 10% polyacrylamide gels and detected by autoradiography.

PCNA, DNA topoisomerases and TdT detection

PCNA. PCNA was detected in Western blotting experiments as described above, using mouse monoclonal anti-PCNA antibodies (clone PC-10; Boehringer Mannheim).

Topoisomerase I. The topoisomerase I activity assay relies on relaxation of supercoiled plasmid DNA by cleavage at the hexameric topoisomerase I consensus sequence cloned into a plasmid DNA substrate (pHOT; Topogen Inc.). Various amounts of nuclear extracts derived from Bcl1.1 day 0 or day 2 p.i. cells were incubated with 0.3 µg DNA substrate for 30 min at 37°C in the appropriate buffer. Reactions were stopped by addition of SDS (to 0.4%) and proteinase K (to 0.1 mg) and incubation at 37°C for 15 min. Products were visualized by agarose gel electrophoresis and ethidium bromide staining.

Topoisomerase II. ATP-dependent decatenation of catenated DNA, i.e. the resolution of multimeric, intertwined kinetoplast DNA circles (*Crithidia fasciulata* KDNA; Topogen Inc.) into monomers was used to assay topoisomerase II activity (50). The KDNA network will not migrate into agarose gels, while the liberated minicircles will. Incubation procedures and product visualization were as described for topoisomerase I except that the buffer contained ATP. Immunoblotting used human or rabbit polyclonal anti-topoisomerase I or II (α-form) antibodies (Topogen Inc.) and 10 µg nuclear extract prepared from wild-type preB cells before or after induction of differentiation.

TdT. Detection of TdT was achieved using anti-TdT rabbit polyclonal antibodies (Supertech, Bethesda, MD) in the Western blotting procedure as described above.

RESULTS

Regulation of DNA polymerase activities and expression upon induction of preB cell differentiation

Cell extracts were prepared and depleted of PCNA from two V(D)J recombination-proficient preB cell lines, Bcl1.1 (*bcl*-2 transgenic) and Pal 1 (non *bcl*-2 transgenic) and from a V(D)J recombination-incompetent *RAG*-2$^{-/-}$ preB cell line (R2B) at different times post differentiation induction. Figure 1A shows the activities of DNA polymerases α, δ and ε at days 0, 1, 2 and 3 p.i. for wild-type cell (Bcl 1.1) nuclear extracts. While the activity levels for pol α and ε remained low or decreased, pol δ increased up to almost 8-fold (on average 4-fold) from ~60 to 460 U/mg protein at day 2. Pol δ activity decreased at day 3 after induction below starting levels. The activity of pol β was also tested and found to gradually decrease to ~50% of starting levels at day 3 p.i. (not shown).

The pattern of pol activities in nuclear extracts derived from *RAG*-2$^{-/-}$ preB cells (R2B) resembled the situation in wild-type cell extracts (Fig. 1B). Like wild-type cells, *RAG*-2$^{-/-}$ preB cells stop proliferation upon induction of differentiation. Pol δ was up-regulated several-fold at days 1 and 2, while pol α and ε remained at their low starting levels or below. As observed in Bcl1.1 cells, the activity of pol δ decreased at day 3 p.i.

For a further control, nuclear extracts derived from Pal 1 cells were assayed in parallel (Fig. 1C). Since these wild-type preB cells enter apoptosis after induction, the enzyme activity can be assayed only up to day 2 p.i., whereafter massive cell death commences. The polymerase activity profiles up to this point, however, reflected those seen in Bcl1.1 and R2B cell extracts.

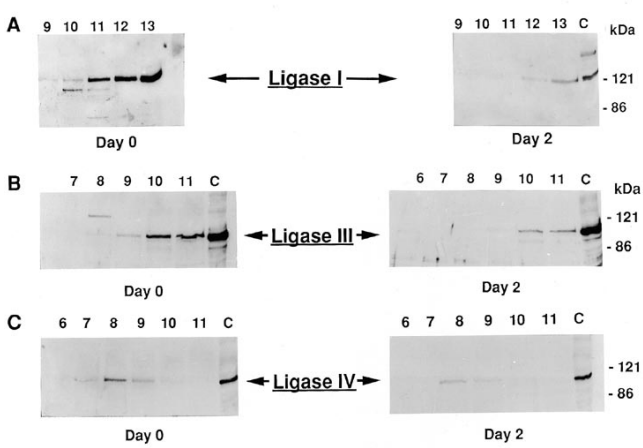

Figure 4. Immunoblot analysis of DNA ligases upon induction of differentiation. (**A**) DNA ligase I was probed in chromatography fractions (numbered at top) derived from day 0 or day 2 p.i. Bcl1.1 nuclear extracts. The position of DNA ligase I (~125 kDa) is marked by an arrow. (**B**) Bcl1.1 day 0 or day 2 p.i. nuclear extract fractions (numbered at top), separated in four identical 7.5% SDS–polyacrylamide gels, were probed with polyclonal anti-DNA ligase III or IV antibody as indicated. Protein amounts in the fractions were: fraction 8, 2.2 (day 0) and 1.0 µg (day2); fraction 10, 5.5 (day 0) and 3.5 µg (day 2). Size markers are to the right (in kDa). C, control HeLa cell nuclear extract fraction (5 µg).

Cytoplasmic extracts, finally, yielded very similar results, with only pol δ activity being enhanced (Fig. 1D).

Pol δ protein expression was also monitored in immunoblotting experiments using anti-pol δ sera (43) for probing total cell lysates of wild-type (Bcl 1.1) cells before (day 0) and up to 4 days after induction of differentiation (Fig. 2). Expression of pol δ was found to remain unchanged for up to 2 days p.i., after which protein levels commence to decrease. The decrease in protein expression kinetically parallels the reduction in activity seen at day 3 p.i. and the completion of V(D)J rearrangements around day 3 p.i.

Expression of PCNA upon induction of preB cell differentiation

Activity of pol δ is dependent on the presence of PCNA (51,52). Using immunoblotting and a monoclonal anti-PCNA antibody, expression of PCNA before and after induction of preB cell differentiation was investigated (Fig. 3). Despite the immediate stop in replication, PCNA levels remained high for 2 days and decreased significantly only at day 3 after induction. The decrease in PCNA levels at day 3 paralleled the reduced activity and protein levels of pol δ observed at this time point. It is also consistent with completion of V(D)J heavy and light chain gene rearrangements after ~2 days differentiation (36,39,40).

Expression and activity of DNA ligases upon induction of preB cell differentiation

Expression of DNA ligases I, III and IV was analyzed by immunodetection methods. Immunoprecipitation of DNA ligase I in nuclear and cytoplasmic extracts of Bcl1.1 cells revealed a clear signal at day 0 in both extracts, but the signal completely disappeared at day 2, indicating a drastic reduction in DNA ligase I protein levels after induction of differentiation (not shown). Western blotting experiments (Fig. 4A) confirmed these results, with a greatly decreased DNA ligase I signal at day 2 p.i.

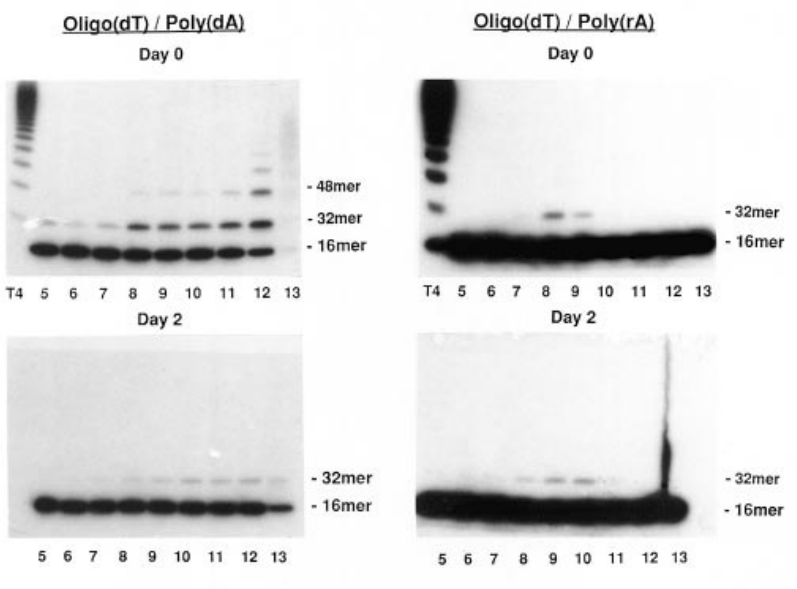

Figure 5. DNA ligase activities before and after induction of preB cell differentiation. DNA ligase activity tested in Bcl1.1 (day 0 or day 2 p.i.) nuclear extract fractions (numbers below each assay). The 16mer substrates are indicated at the top; the products are multimers (32mer or 48mer). T4, T4 DNA ligase control reaction (0.2 U).

in chromatography fractions obtained from Bcl1.1 cells and enriched for that DNA ligase. Separation of DNA ligases III and IV (47) was achieved by ion exchange chromatography of Bcl1.1 cell nuclear extracts and the DNA ligase protein levels in corresponding fractions were assayed by Western blotting (Fig. 4B and C). Protein levels of both DNA ligases appeared modestly decreased at day 2 p.i., as can be seen for DNA ligase III in fractions 9–11 and for DNA ligase IV in fractions 7–9. However, the amount of day 2 derived protein loaded for gel electrophoresis was approximately half of that used for day 0. Therefore, the true levels of DNA ligases III and IV do not change significantly at day 2, contrasting with the results obtained for DNA ligase I.

Activity assays were carried out for all three DNA ligases using the above-mentioned chromatography fractions (Fig. 5). Distinct substrate specificities allowed further distinction between the DNA ligases. While oligo(dT)·poly(dA) is ligatable by all three DNA ligases, ligase I, in contrast to III and IV, does not accept oligo(dT)·poly(rA) substrates. Consequently, activity of a certain protein fraction observed with the latter substrate should reside in either DNA ligase III or IV. The 16mer oligo(dT)·poly(dA) substrate was efficiently converted into 32mer and 48mer products by fractions 5–13 derived from day 0 extracts. Day 2 p.i. derived fractions, however, displayed much less activity. No product formation was seen with fractions 5 and 6, but a relatively weak 32mer product signal was observed in fractions 7–13. Thus, overall ligase activity on this substrate, acceptable to all three DNA ligases, decreased significantly and, moreover, the loss of activity correlated with a reduction in DNA ligase I protein in the corresponding day 2 p.i. fractions (Fig. 4). The remaining activity was strongest in fractions 8–12, which were shown to contain DNA ligases III (fractions >10) and IV (fractions 8 and 9; Fig. 4). The oligo(dT)·poly(rA) substrate was converted into product only by fractions 8 and 9 in day 0 derived extracts. At day 2 after induction, the total amount of product formation from the oligo(dT)·poly(rA) substrate remained unchanged, but the activity peaks appeared to be shifted towards the fractions enriched for DNA ligase III (fractions 9–11). This could be explained by a mild reduction in DNA ligase IV activity, present predominantly in fractions 8 and 9, with a simultaneous increase in DNA ligase III activity, enriched in fractions 9–11.

Taken together, the data indicate down-regulation of DNA ligase I, but no significant changes in DNA ligase III and IV activities after induction of differentiation.

Activity and expression of topoisomerases I and II and TdT upon induction of preB cell differentiation

As DNA metabolic enzymes have not directly been implicated in V(D)J recombination, but which nevertheless might be regulated at the onset of cell differentiation, topoisomerases I and II were investigated. Expression of the enzymes in cytoplasmic and nuclear extract fractions was monitored by immunoblotting using polyclonal antibodies against either topoisomerase I or IIα. For both enzymes, protein levels did not change significantly during the first 2 days and decreased by ~30% at day 3 p.i. (not shown).

Enzyme assays for topoisomerases I and II were performed on nuclear extracts from wild-type and *RAG-2*$^{-/-}$ preB cells before (day 0) and after (day 2) induction of differentiation. Titration experiments showed similar topoisomerase II-mediated KDNA minicircle product formation by day 0 and day 2 derived extracts (Fig. 6A, left). For topoisomerase I (Fig. 6A, right), decreasing amounts of nuclear extract protein derived from Bcl1.1 cells either before or at day 2 after induction were added to the reaction. No difference in activity between day 0 and day 2 p.i. could be observed (lanes 1–4 and 5–8). With the highest amount of protein, limited DNA degradation occured. In sum, no gross alterations in topoisomerase I and II activities were observed.

The only enzyme known to act specifically at a late step in V(D)J recombination is TdT (18). TdT is expressed exclusively in early stage lymphocytes (reviewed in 5). We monitored TdT expression in nuclear extracts by immunoblot analysis before and up to 4 days after induction of preB cell differentiation (Fig. 6B).

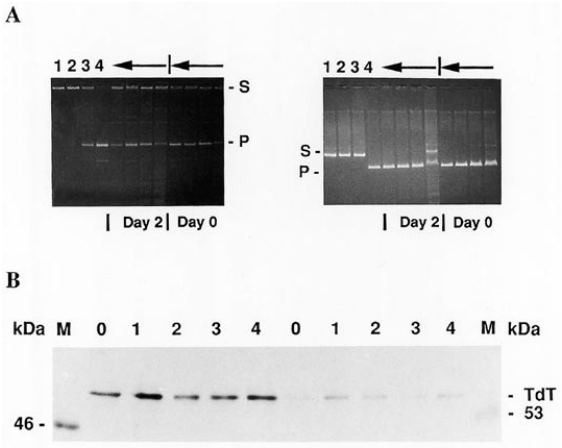

Figure 6. (**A**) Topoisomerase I and II activities in preB cells before and after induction of differentiation. Activities of topoisomerases I (right) and II (left) were determined in day 0 and day 2 p.i. derived Bcl1.1 nuclear extracts. Arrows indicate serial dilutions (2.4, 1.2, 0.6 and 0.3 µg protein/reaction). Lane 1, untreated substrate DNA; lane 2–4, 0.08, 0.4 and 4 U topoisomerase I or II respectively. S, substrate; P, product. (**B**) Immunoblot analysis of TdT before and after induction of differentiation. 15 (left) or 3 µg (right) total cell lysate protein were separated by 7.5% SDS–PAGE. Numbers at the top refer to days after induction. The position of TdT is indicated.

To allow accurate quantification, two different amounts of total cell extracts were loaded (15 µg, left, and 3 µg, right). As expected, the enzyme is present at day 0. At day 1 p.i., expression of the protein was increased ~2-fold, whereafter it decreased below starting levels.

DISCUSSION

The advanced steps in V(D)J recombination are characterized by the action of several DNA metabolic enzymes such as TdT, DNA polymerase(s), nucleases and DNA ligase(s). Specificity of V(D)J recombination is provided at the initiation of the reaction by RAG-1 and RAG-2 proteins (10,12). Except for TdT, none of the other enzymes may act exclusively in V(D)J recombination and they may be recruited from the general DNA metabolic enzyme pool. There might, however, be only one member of each individual enzyme family, such as the DNA polymerases or DNA ligases, which is able to perform the function required to process V(D)J coding and signal joins.

Among the DNA polymerases, we found that pol δ activity increased significantly after the onset of preB cell differentiation. Pol α, β and ε remained at low levels or even decreased. Concurrently, pol δ protein expression levels stayed high for ~2 days p.i. before diminishing. Since no significant increase in pol δ protein levels was observed at day 1 and day 2 p.i., up-regulation of pol δ activity is likely to be a post-translational event. The increase in pol δ activity, which is the major replicative DNA polymerase already present at high levels in proliferating preB cells, is even more remarkable, since the cells arrest and stop replication immediately after withdrawal of IL-7. This up-regulation renders pol δ an interesting candidate to be responsible for DNA synthesis associated with V(D)J recombination. In the V(D)J recombination process, a DNA polymerase is needed to fill in single-stranded regions generated by opening of hairpin intermediates, by exonucleolytic degradation at the coding ends and by addition of N-nucleotides by TdT (reviewed in 6,9,11). Pol δ has the properties to fill in such short gaps completely (24).

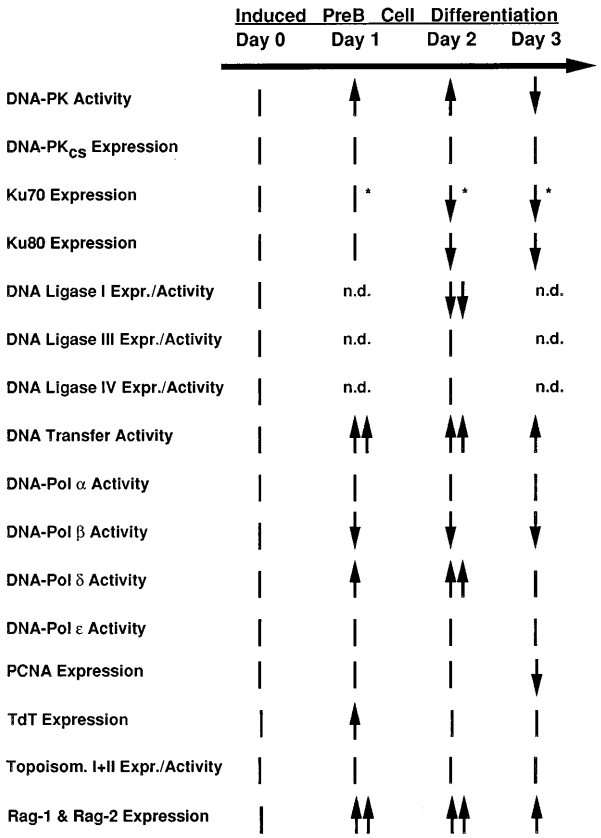

Figure 7. Overview of DNA metabolic proteins in preB cell differentiation. Data are derived from this and an earlier study (40). Up- or down-directed arrows indicate up- or down-regulation of the protein (expression and/or activity); | indicates no change; n.d., not determined; * indicates appearance of a new anti-Ku70 cross-reacting polypeptide.

The increase in pol δ activity was not only observed in preB cell lines which are wild-type with respect to their ability to rearrange their immunoglobulin loci. It was also found in extracts derived from a V(D)J recombination-incompetent *RAG-2*$^{-/-}$ preB line. Other studies, however, have demonstrated that R2B cells undergo all developmental changes normally associated with preB cell differentiation (39,40) except V(D)J recombination. These cells can even be differentiated to a later stage and induced to perform class switch recombination (Rolink *et al.*, submitted for publication). Thus it seems very likely that up-regulation of pol δ is part of a developmental program normally correlated with V(D)J recombination, but not dependent on succesful initiation of V(D)J recombination. Since the activity of pol δ depends on PCNA (51,52), we examined expression of this protein during differentiation. PCNA expression is often tightly linked to progression through the cell cycle, so that its expression has been used as a marker for the actively replicating state of cells (53,54). The situation in developing preB cells, however, differs. Though the cells arrest and enter G_0 immediately after induction, PCNA protein levels stay high for ~2 days. Thus, in preB cells there is sufficient PCNA available when pol δ is stimulated in the G_0 stage. The nuclear presence of PCNA throughout all phases of the

cell cycle has also been demonstrated in UV-irradiated cells (55,56), indicating a role of the protein in DNA repair, which was confirmed in cell-free DNA repair assays (57). Gap filling DNA synthesis in these repair reactions resembles DNA synthesis associated with V(D)J recombination.

Of the four known mammalian DNA ligases, our studies mark DNA ligase I as the most unlikely candidate to participate in V(D)J recombination. Protein levels and enzyme activities dropped significantly after the onset of differentiation. Since DNA ligase I became known as the major replicative DNA ligase in many cell types (49,58), it is not surprising to see the enzyme level decreasing in non-proliferating cells. DNA ligase III has most often been implicated in DNA recombination and repair (26,31,33), while the function of the recently discovered DNA ligase IV is still unknown (34). Our results indicate moderately reduced expression or stability of DNA ligase III mRNA (not shown), but protein levels of both ligases III and IV did not change significantly after induction of preB cell differentiation. The half-life of DNA ligase III and IV proteins in differentiating lymphocytes is unknown, but from our data it is plausible that the amount of protein stays nearly unchanged over a period of 2 days, providing ligation activity for the cells.

In ligation assays the activity of DNA ligase I, assayed with the oligo(dT)·poly(dA) substrate, was markedly reduced after the onset of differentiation. This contrasts with DNA ligase III and IV activities, which are not down-regulated. Product formation from the oligo(dT)·poly(rA) substrate slightly increased in the protein fractions enriched for DNA ligase III and did not change significantly in DNA ligase IV-enriched fractions after 2 days p.i. However, as there are only modest differences between DNA ligases III and IV in their response to induction of preB cell differentiation, we cannot rule out either of them as a candidate for sealing V(D)J recombination joints.

No changes were observed in activities of topoisomerases I and II, concurrent with largely unaltered levels of topoisomerase I and IIα proteins. Both topoisomerases are abundant enzymes involved in many cellular processes, including transcription, replication, recombination and repair, and topoisomerase II has an additional function in maintaining the chromosomal structure through its association with chromatin scaffold components (reviewed in 59). This multitude of functions makes largely unaltered levels for these enzymes even after differentiation and cell arrest plausible. Based on current knowledge of the V(D)J recombination mechanism, a requirement to relieve topological stress comparable with that generated during replication, transcription or unwinding associated with homologous pairing of DNA duplexes seems unlikely.

Though TdT is expressed in early B lymphocytes (for review see 5) and participates in creating antigen receptor diversity by adding N-regions to the coding ends, the enzyme is not essential for completion of the V(D)J recombination reaction (60,61). Our results show TdT expression before and after induction of differentiation and thus availability of this activity for V(D)J rearrangements. There is a moderate increase in expression early after the onset of differentiation (day 1), at a time when light chain rearrangements occur in this system (35). Thereafter, expression decreases. The presence of TdT protein at the proliferative state of preB cells (day 0) can be explained by expression in the preceding differentiation step at the proB cell stage, where D_H to J_H rearrangements occurred and the enzyme has been shown to be present. It is only after μ heavy chain gene V(D)J rearrangements have been completed that TdT expression starts to be down-regulated (5,35,36).

An overview of the results presented here in combination with a previous study on different DNA metabolic proteins (40) is given in Figure 7. These observations might be helpful for our understanding of (i) regulation of DNA metabolic enzymes in association with lymphocyte development, (ii) V(D)J recombination *in vivo* and consequently (iii) for *in vitro* reconstitution of the complete V(D)J recombination reaction. Finally, (iv) they also provide insights into the regulation of DNA metabolic enzymes and proteins upon cell cycle arrest, which is coupled to cell differentiation processes.

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