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Accelerated Publications

Terminal Deoxynucleotidyltransferase Containing Megadalton Complex from Young Rat Thymus Nuclei: Identification and Characterization†

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ABSTRACT: Nuclear matrix prepared from 2–3 week old rat thymuses contains tightly bound TdT activity which has been quantitatively solubilized with nonionic detergent and sonication. TdT is contained in a discrete complex with a sedimentation value of 23 S. The complex is retained on an anti-TdT antibody column and contains DNA ligase and 3′–5′ exonuclease activities as well as DNA and several other proteins but is devoid of replicative DNA polymerases. Such a type of multienzyme complex is absent from the nuclear extracts of thymus prepared from older rats and also from liver and spleen extracts of young and old rats.

Terminal deoxynucleotidyltransferase (TdT;[1] EC 2.7.7.31) is a unique DNA polymerase that catalyzes the polymerization of deoxyribonucleotides onto the 3′-OH end of DNA without template direction (Bollum, 1974). The restricted presence of TdT in prelymphocytes implies that TdT is closely related to lymphocyte development in both thymus gland and bone marrow (Baltimore, 1974; Chang & Bollum, 1987). Extensive biochemical characterization including active site determination of TdT has been carried out (Bollum, 1974; Bhalla et al., 1977; Modak, 1978, 1979; Modak & Gillerman-Cox, 1982; Pandey & Modak, 1987a,b, 1988a,b, 1989; Chang & Bollum, 1987). However, its biological role remains to be elucidated. Recent studies have provided some evidence to suggest that TdT may be involved in immunoglobulin (Ig) or T cell receptor (TCR) gene rearrangement/recombination to generate the diversified immune response observed in vertebrates (Alt & Baltimore, 1982; Desiderio et al., 1984; Roth et al., 1986; Alt et al., 1987). The process of TdT-mediated addition of nonconserved sequences, however, is only one of many steps in the process of recombination, and hence, the concerted action of several enzymes/proteins may be required at the site of rearrangement/recombination. In this context, various components may be expected to exist in the form of a multienzyme/protein complex. In addition, logistically one may not expect TdT to be free in the nucleoplasm since it has the

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[1] Abbreviations: TdT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphates; DTT, dithiothreitol; PMSF, phenylmethane sulfonyl fluoride; Ig, immunoglobulin; TCR, T cell receptor; VDJ, variable, diversity, and joining segments of Ig or TCR genes; NP40, Nonidet P40; SDS, sodium dodecyl sulfate.

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potential to add nucleotides at any 3′-OH terminus, which is also used in DNA replication. Nearly 2 decades ago, a nuclear membrane matrix from calf thymus nuclei was indeed found to contain both DNA polymerase and TdT activities (Yoshida et al., 1971). We have therefore begun a reinvestigation of TdT distribution in the nuclei of young rat and have found that a significant amount of nuclear TdT remains tightly associated with the high salt resistant nuclear matrix (Pandey et al., 1989). This observation suggested that the matrix-associated TdT may be contained in a complex consisting of various proteins/enzymes. We report here the solubilization and characterization of matrix-bound TdT in rat thymus gland that appears to exist as a multiprotein/multienzyme complex with a molecular mass of approximately 1.3×10^6 daltons. This is the first time that a complex of this nature containing TdT has been shown to exist. The exclusive presence of this complex in young thymic nuclei, the overall properties of this complex, and the nature of the associated enzyme activities imply that this complex may be a part of the putative recombinase system involved in Ig/TCR gene recombination.

Materials and Methods

Isolation of Thymus Nuclei and Nuclear Matrices. Thymus glands excised from 100 rats (2–3 week old; Wistar strain) were immediately transferred to ice-cold 0.25 M sucrose in 5 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$, 1 mM DTT, and 1 mM PMSF. The glands were minced, and nuclei were isolated as described by Berezney and Coffey (1974). Nuclear matrix from thymus nuclei was prepared by a standard procedure as described before (Smith & Berezney, 1982; Pandey et al., 1989). The purified nuclei were suspended so as to contain 2 mg of DNA/mL in 0.25 M sucrose buffer and were incubated overnight at 4–5 °C in the presence of 0.5 μg of DNase/mg of nuclear DNA. The nuclease-digested nuclei were then extracted with buffer containing 10 mM Tris-HCl (pH 7.4), 1 mM PMSF, 1 mM DTT, 0.2 mM $MgCl_2$, and 50 mM NaCl followed by 0.5 and 1 M NaCl in the same buffer. It is important that extractions with low- and high-salt buffers be carried out in a Teflon tissue homogenizer to avoid any clump formation. This procedure removes approximately 85 and 95%, respectively, of total nuclear protein and DNA and approximately 85–90% of total nuclear TdT. The nuclear matrix contains 10–15% of the total TdT activity after the above extractions. Nuclear matrix was stored in buffer containing 50% glycerol at − 70 °C.

Preparation of Solubilized Matrix Extract and Sucrose Gradient Analysis. An aliquot of matrix suspension was mixed with 2 volumes of buffer containing 1.5 M NaCl and 0.15% NP40. The suspension was homogenized and subjected to a brief sonication (30-s pulse at lowest setting, Lab-Line Ultratip Labsonic system, Lab-Line Instruments Inc.) and centrifuged at 13000*g* for 15 min. The solubilized matrix was carefully removed and loaded onto 5–20% sucrose gradients prepared in a buffer containing 1 M NaCl. Gradients were centrifuged at 1.55×10^5 g for 6 or 16 h in an SW 40 rotor, and 24 equal fractions were collected. An aliquot was assayed for TdT activity. Sedimentation of the marker proteins, bovine serum albumin (4.4 S), catalase (11.2 S), and thyroglobulin (19.2 S), indicated that the 16-h gradient was isokinetic. The S values of these markers are indicated on the scale above the sucrose gradient profiles (see figures). Sedimentation of 70S *Escherichia coli* ribosome and 30S and 50S ribosomal subunits also indicated approximately isokinetic migration in 6-h gradients.

Assay of TdT, DNA Polymerases, 3′–5′ Exonuclease, and DNA Ligase. The standard assay conditions for TdT and DNA polymerase assays have been described before (Bhalla et al., 1977; Modak, 1978). Activated calf thymus DNA or oligo(dA)$_{10}$ and [^{3}H]dGTP (40 μM; 800 cpm/pmol) were used as the primer and substrate with Mn^{2+} (1 mM) as the divalent cation.

The assay for 3′–5′ exonuclease activity was carried out essentially as described before (Pandey & Modak, 1988b) with poly(dA)·[^{32}P](dT)$_{12-18}$ as the substrate. Exonuclease activity was measured by a newly developed sensitive assay procedure (V. P. Dave et al., unpublished results). Briefly, oligo(dT)$_{10}$ is labeled at its 5′ end with [γ-^{32}P]ATP and T_4 polynucleotide kinase and is annealed to poly(dA) in a nucleotide mole ratio of 2:3. The annealed poly(dA)·[5′-^{32}P](dT)$_{10}$ is used as the ligase substrate in conjunction with the Klenow fragment of *E. coli* DNA polymerase I and nonradioactive dTTP as the gap-filling component. In this reaction, labeled oligo(dT) primers are extended by the Klenow fragment until the 5′ end of the next primer molecule. The adjacent 3′ and 5′ ends of the two primers are joined by DNA ligase. The labeled 5′ end of the primer molecule thus becomes an internal residue and is not cleaved by alkaline phosphatase. The insensitivity of the above product to alkaline phosphatase thus provides a sensitive assay to determine the extent of ligation. A typical reaction mixture for the ligase assay contains 50 mM Tris-HCl, pH 7.8, 0.5 μg of poly(dA)·[5′-^{32}P](dT)$_{10}$ (2×10^5 cmp), 100 μM dTTP, 0.5 mM ATP, 10 mM $MgCl_2$, 1 mM DTT, 1 unit of Klenow fragment of *E. coli* pol I, and the desired aliquot of sample containing DNA ligase activity in a final volume of 100–200 μL. After incubation at 37 °C for 30 min, 5 units of bacterial alkaline phosphatase (Sigma Chemical Co.) was added to each reaction mixture, and incubations were continued at 68 °C for 30 min. The phosphatase reaction was stopped by addition of 10% cold TCA, and the extent of ^{32}P radioactivity rendered resistant to alkaline phosphatase due to ligation was measured in the TCA-insoluble material.

Affinity Purification of TdT Complex. The low-speed supernatant of ascites fluid enriched in anti-calf TdT monoclonal antibodies was a generous gift from Mary Sue Coleman of the University of Kentucky Medical Center. An immunoaffinity column consisting of TdT monoclonal antibody covalently linked to protein A–Sepharose was prepared as described by Robbins et al. (1987). TdT activity sedimenting at the 23S position of the gradient was concentrated and desalted, in a Centriprep-10 ultrafiltration device, to a final salt concentration of 0.15–0.2 M. The concentrated sample was mixed with 3 mL of monoclonal TdT antibody–protein A–Sepharose matrix, stirred for 2 h at 4 °C, and poured into a 1.5 × 5 cm column. The column was washed with 200 mL of 50 mM Tris-HCl, pH 7.4, containing 0.2 M NaCl, 1 mM DTT, and 0.2 mM $MgCl_2$ followed by a similar wash with buffer containing 1 M NaCl. The TdT activity bound to the antibody column was eluted with 2 M $MgCl_2$ in the same buffer. Since a high Mg concentration was found to be unfavorable for complex stability, the 2 M $MgCl_2$ eluate was collected directly in a tube that contained four volumes of dilution buffer (50 mM Tris-HCl, pH 7.4, 200 mM NaCl, and 1 mM DTT). The content of the tube was then transferred to the Centriprep-10 ultrafiltration device, concentrated, and either resolved on a linear 5–20% sucrose gradient (see Figure 4) or stored in 50% glycerol at −70 °C.

SDS–Polyacrylamide Gel Electrophoresis and Immunoblotting. An aliquot of concentrated sample equivalent to 50–100 μg of protein was solubilized in protein solubilizing buffer and subjected to SDS–polyacrylamide gel electrophoresis (Laemmli, 1970) using a 10–20% precast polyacrylamide

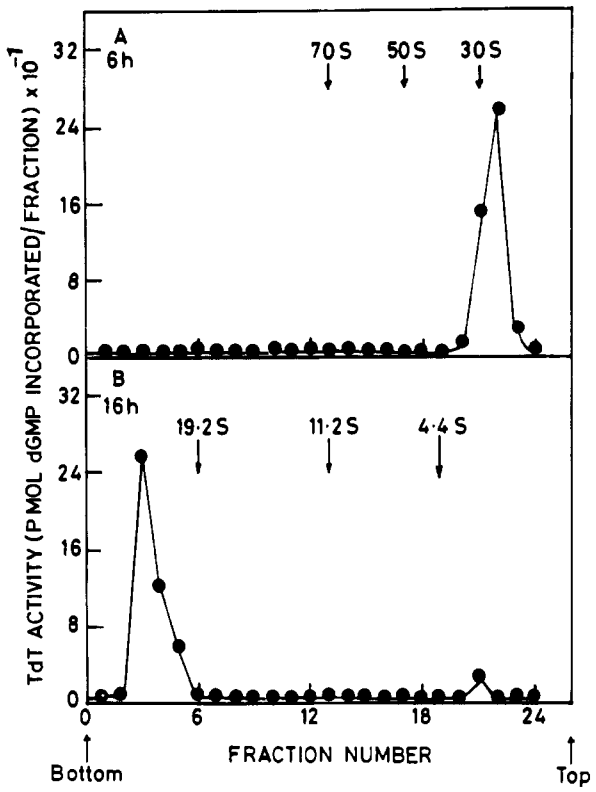

FIGURE 1: Sucrose gradient analysis of TdT activity solubilized from rat thymus matrix. Thymus nuclear matrix extract prepared from 2–4 week old rats was centrifuged on linear 5–20% sucrose gradients containing 1 M NaCl at $1.55 \times 10^5 \times g$ for either 6 (A) or 16 h (B). Gradients were fractionated from the bottom, and an aliquot from each fraction was assayed for TdT activity with [^{3}H]dGTP as the substrate and activated DNA as the initiator/primer (see Materials and Methods). Results are expressed as picomoles of dGMP incorporated per gradient fraction in 15 min. The positions of standards of known S values, run in parallel gradients, are indicated on the gradient profiles.

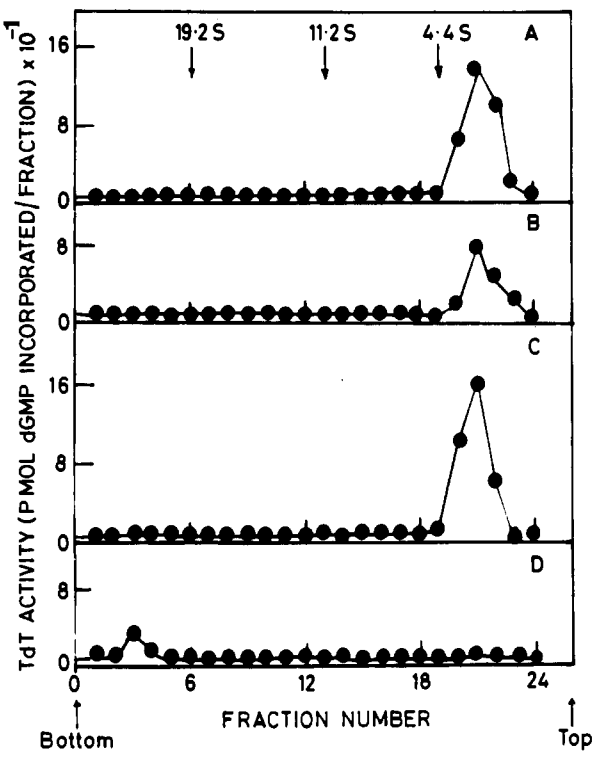

FIGURE 2: Sucrose gradient analysis of TdT activity from thymus glands of 25 week old rats. Aliquots of the extracts containing TdT activity from various fractions were processed on 5–20% sucrose gradients as described in Figure 1 for 16 h. Sedimentation pattern of TdT obtained is for (A) cytosol extract, (B) low-salt extract of nuclei, (C) 1 M NaCl extract of nuclei, and (D) solubilized nuclear matrix extract.

linear gradient gel (obtained from Integrated Separation Systems, Hyde Park, MA). Following electrophoresis, proteins were either stained with Coomassie blue according to a standard protocol or transferred to nitrocellulose membranes for immunoblotting. The antibody used for the detection of TdT was affinity-purified rabbit anti-calf TdT obtained from Bethesda Research Labs.

Results

Nuclear Matrix Bound TdT and Its Solubilization. The nuclear matrix prepared from the exogenous and endogenous nuclease digestion contained 10–15% of the total TdT activity present in the nuclear extracts, prepared from pooled thymus glands obtained from 100 2–3 week old rats. The majority of matrix-bound TdT activity is solubilized by inclusion of 0.1% NP40, a nonionic detergent, in the high-salt buffer together with brief sonication. The soluble matrix extract contained approximately 80 and 70% of the matrix-bound protein and DNA, respectively. The use of nonionic detergent alone effected some (~20%) release of matrix-bound TdT; however, high salt together with detergent and sonication is required for nearly quantitative release of TdT from the nuclear matrix. An approximate quantity of DNA ligase and 3′–5′ exonuclease activity in the final solubilized fraction is estimated to be between 1 and 3% of the total activity present in nuclear extracts.

Molecular Size of TdT Complex Released from Matrix. In order to determine the approximate molecular mass of TdT solubilized from the nuclear matrix, sucrose density gradient centrifugation of the soluble matrix extract was carried out. Gradients were centrifuged for 6 and 16 h, respectively, to resolve the components sedimenting at high and low rates. The results shown in Figure 1 clearly demonstrate that the majority of the TdT activity solubilized from the matrix sediments at the 23S position, which corresponds to a molecular mass of approximately 1.3 Mdalton. A small but reproducible amount of TdT was also detected in fractions sedimenting at the 3S position, which corresponds to a molecular mass of approximately 55–60 kDa. The molecular weight of TdT that sedimented at the 23S and 3S positions was determined from a standard curve obtained from S value versus molecular weight of known protein markers. The mean sedimentation value ± SE of TdT in complex form and in free form determined from three different preparations of solubilized matrix extracts were 22.9S ± 0.6S and 2.5S ± 0.5S, respectively. For simplicity, we have designated these as 23S and 3S when referring to the megadalton complex of TdT and free TdT, respectively. TdT activity present in low-salt and high-salt extracts of nuclei consistently sedimented at the 3S position (data not shown).

Other Enzyme Activities Associated with 23S TdT Complex. Since TdT has been implicated in TCR/Ig gene rearrangement/recombination, it was of interest to investigate if other enzyme activities, presumably associated with the putative recombinase system, are contained in the complex. Each gradient fraction was therefore assayed for TdT, DNA ligase, 3′–5′ exonuclease, and DNA polymerase activities. The sedimentation profiles of those enzymes detected in the fractions are shown in Figure 3. A major peak of DNA ligase and 3′–5′ exonuclease activity cosedimented with TdT at the 23S position of the gradient. No replicative DNA polymerase activities were seen in the 23S complex as judged by the following characteristics of DNA synthesis: (a) it was significantly

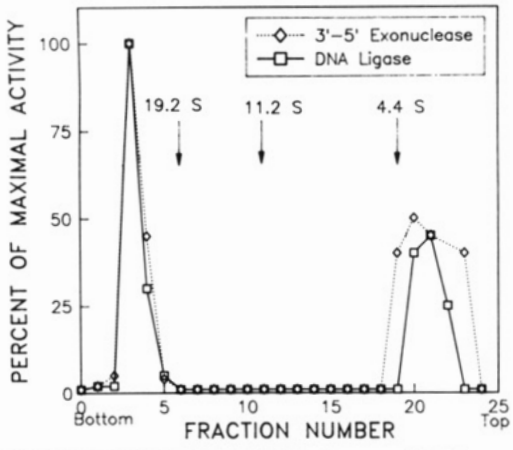

FIGURE 3: Sedimentation profile of DNA ligase and 3′–5′ exonuclease activities present in solubilized nuclear matrix. The gradient fractions from the 16-h run (Figure 1B) were assayed for DNA ligase and exonuclease activities with assays described under Materials and Methods.

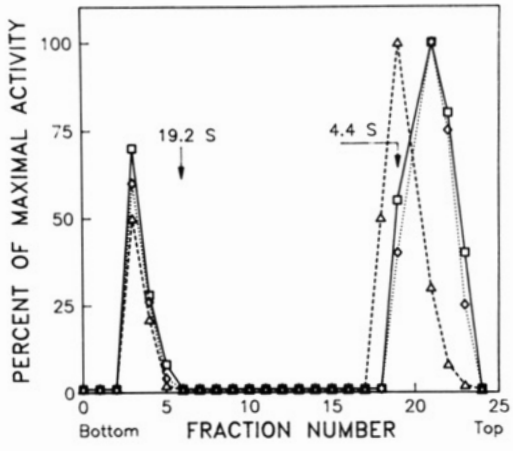

FIGURE 4: Sedimentation profile of solubilized TdT complex and associated enzyme activities purified by an immunoaffinity column. The fractions containing TdT activity at the 23S position (Figure 1B) were adsorbed onto a monoclonal TdT antibody column, eluted with 2 M $MgCl_2$, desalted, and concentrated with a Centriprep-10 rapid ultrafiltration device (see Materials and Methods). An aliquot of the concentrated fractions was reanalyzed on a 5–20% linear sucrose density gradient as described in Figure 1. The gradient fractions were assayed for TdT, DNA ligase, and exonuclease activities. The maximum [^{3}H]dGMP incorporation due to TdT activity was 102 pmol/gradient fraction. The maximum ^{32}P radioactivity of poly(dA)·[5′-^{32}P](dT)$_{10}$ rendered resistant to alkaline phosphatase by DNA ligase was 18×10^3 cpm per assay. The maximum ^{32}P radioactivity of poly(dA)·[3′-^{32}P]oligo(dT) rendered susceptible due to 3′–5′ exonuclease was 31×10^3 cpm per assay under standard assay conditions.

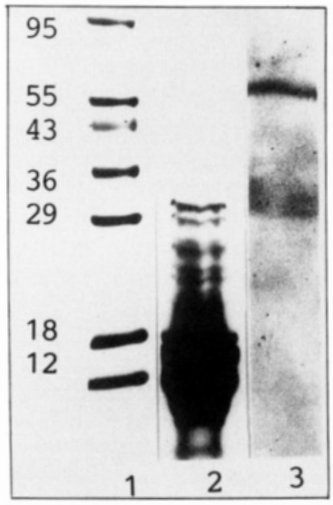

FIGURE 5: SDS–polyacrylamide gel profile of TdT antibody purified complex. An aliquot of TdT complex (100 μg of protein) purified by the TdT antibody column was dialyzed to remove $MgCl_2$ and then applied to a 10–20% polyacrylamide gradient gel in duplicate. Prestained molecular weight markers were simultaneously run. One of the sample lanes was transferred to nitrocellulose paper, and immunoblotting was performed with anti-TdT antibody while the duplicate sample was stained with Coomassie blue. Lane 1 shows molecular mass markers, lane 2 shows Coomasie blue staining pattern, and lane 3 shows immunoblot of the sample with TdT antibody.

reduced in the presence of all four dNTPs; (b) it was insensitive to aphidicolin; (c) it was strongly inhibited by NEM; (d) no activity was noted with the poly(dA)·oligo(dT) and TTP template-primer system. We therefore conclude that the DNA synthesis catalyzed by the 23S complex is solely due to TdT activity.

Age-Dependent Existence of 23S Multienzyme Complex and Its Tissue Specificity. Functional differentiation and development of prelymphocytes is known to be an age-dependent phenomenon that seems to correlate well with thymic involution (Sujimoto & Bollum, 1979). It was therefore important to determine if a multienzyme megadalton TdT complex was also present in the thymus of older animals. Results obtained from the nuclear matrix prepared from 100 thymuses from 6 month old rats (Figure 2) clearly show that no TdT can be detected in a high molecular weight complex form. Soluble nuclear extracts (nonmatrix fraction) did contain a significant quantity of TdT that sedimented at 3S (Figure 2). Similar experiments were carried out with solubilized nuclear matrix extracts from liver and spleen nuclei of young (2–4 weeks) and old (25 weeks) rats to determine whether the 23S multienzyme complex is unique to thymus tissue in young rats. The results obtained from sucrose gradient analyses clearly showed the absence of such a multienzyme complex from liver and spleen nuclei of both the young and old rats (data not shown).

Purification of TdT Complex by Anti-TdT Antibody Column. In order to absolutely confirm that the observed megadalton complex was indeed a true TdT complex, we reasoned that it must bind to an anti-TdT antibody column. Therefore, a column containing protein A–Sepharose-linked monoclonal TdT antibody was used in the purification of TdT solubilized from the nuclear matrix. TdT activity was selectively retained on the column and required 2.0 M $MgCl_2$ for elution. When the dialyzed eluate was subjected to sucrose density gradient centrifugation, about 30–40% of the TdT activity sedimented at the 23S position whereas approximately 60–70% of the enzyme remained at the top of the gradient (Figure 4). Most interestingly, DNA ligase and 3′–5′ exonuclease activities were also retained on the anti-TdT antibody column, and their sedimentation profiles were identical with that of TdT (Figure 4). The recovery of DNA ligase and exonuclease activities in the fraction bound to the antibody column was approximately 30% of the amount applied to the column.

General Properties of the 23S TdT Complex. The affinity-purified 23S TdT complex contained significant endogenous primer/initiator activities when assayed in the absence of exogenous DNA with [^{3}H]dGTP as the substrate (data not shown). These studies indicated that the 23S complex contains tightly associated DNA fragments that can also serve as the initiator for DNA synthesis. The DNA and protein contents in the fractions were determined, and the ratio of protein to DNA was estimated to be approximately 5:1. The size of the DNA associated with the complex was determined by alkaline sucrose gradient centrifugation and was estimated to be

200–300 bases long (data not shown).

SDS–polyacrylamide gel electrophoresis of the affinity-purified TdT complex showed several polypeptides of lower molecular mass (Figure 5) ranging from 10 to 40 kDa with 12–16-kDa proteins as the major components. It was intriguing that the megadalton TdT complex is devoid of polypeptides of high molecular mass, as judged by gel analysis. Although significant amounts of various enzyme activities, including TdT, are associated with the 23S complex, their concentrations appear to be rather low. This is evident from the anti-TdT rabbit antibody immunoblot results where a TdT band corresponding to 58 kDa is clearly seen, but it could not be detected on the stained gel. It should also be noted that the observed smaller polypeptides ranging from 10 to 40 kDa are likely to be part of the structural protein frame required for the association of various functional components of the complex and their interaction with the DNA in the nuclear matrix.

Discussion

This is the first paper that describes the presence of TdT in a soluble multienzyme complex that is derived from the nuclear matrix of young rat thymuses. The megadalton molecular mass of the complex was demonstrated by sucrose gradient analyses in high salt. In addition we have also presented evidence that the TdT-containing complex is retained on anti-TdT antibody columns. Therefore, the existence of a multicomponent complex consisting of TdT, exonuclease, DNA ligase, and other as yet unidentified proteins and DNA seems real and cannot be attributed to a nonspecific aggregation of nuclear proteins and DNA. The fact that replicative DNA polymerases are not associated with the soluble complex also suggests that the complex is not a fortuitous aggregation of proteins that have affinity for DNA. The observations that an extremely low level of complex-bound TdT is found in nuclear matrix preparations of older rat thymus and that there is a total absence of such a complex in spleen and livers of both young and old rat nuclei strongly indicate a thymus-specific location and function for such a complex.

Furthermore, in young animals, the thymus plays a major role in differentiation of T cells, which includes rearrangement or recombination of gene sequences related to VDJ regions (Yancopoulos & Alt, 1985). Joining of variable-region gene segments involves a multistep nonreciprocal recombination process that may include recognition of specific sequences, a precise break between the elements to be joined, addition of de novo nonconserved bases of specific length, and precise ligation leading to deletion of intervening DNA as a circle or to its inversion depending upon the chromosomal orientation of the involved segments (Alt & Baltimore, 1982; Alt et al., 1987). In this context the present finding that a multienzyme megadalton complex consisting of TdT, DNA ligase, 3′–5′ exonuclease, and many other unidentified enzymes/proteins exists on the nuclear matrices of actively differentiating lymphocytes is significant in that it may represent a part of the putative recombinase system involved in TCR/Ig gene rearrangement/recombination in prelymphocytes. It is possible that some of the proteins present in the complex, whose identity has not yet been established, may be components of such a recombination system. Alternatively, these proteins together with complex-associated DNA may form a structural skeleton for the assembly of the active recombinase system. At present it has not been possible to demonstrate in vitro recombination of VDJ segments. However, it is our hope that with additional components and factors, perhaps from soluble extracts, it may be possible to reconstruct such a system.

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