Nuclear Matrix Bound V(D)J Recombination Activity in Rat Thymus Nuclei: An in Vitro System†

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ABSTRACT: We report here that a high level of V(D)J recombination activity is tightly associated with high-salt-resistant nuclear matrix isolated from thymus glands from 2- to 3-week-old rats. The soluble nuclear fractions either were devoid of or contained a very low level of recombinase activity. This is the first time that the process mimicking V(D)J recombination has been achieved in an in vitro system. The matrix-bound V(D)J recombinase activity was further found to be lymphoid specific, detectable only during early stages of development. These observations suggest that in vitro recombination of V(D)J segments of genes encoding antigen-binding proteins could be a matrix-bound process and that the nuclear matrix may be an important intranuclear domain for the functional organization of the V(D)J recombinase system.

The gene encoding variable domains of antigen-binding proteins of the vertebrate immune system is assembled from arrays of variable (V),[1] diversity (D), and joining (J) segments during lymphoid differentiation (Tonegawa, 1983; Hood et al., 1985; Kronenberg et al., 1986; Alt et al., 1986; Yancopoulos & Alt, 1986). Each of the V(D)J segments is flanked by recombinational signal sequences (RSSs) consisting of a conserved palindromic heptamer, a spacer of 12 or 23 nonconserved base pairs, and a conserved AT-rich nonamer (Early et al., 1980; Sakano et al., 1980). These segments are brought together by site-specific recombination to form a large number of unique functional coding sequences at the TCR/Ig loci from much smaller component sequences (Blackwell & Alt, 1989). Although a great deal of information exists on the structure of V(D)J segments and their in vivo rearrangement and recombination in pre-B cell lines (Blackwell & Alt, 1989), it has not been possible to either demonstrate or reconstitute an in vitro recombination system. Moreover, information concerning the biochemical mechanism and the enzymatic machinery involved in the V(D)J recombination is still obscure. Recently, Baltimore and his colleagues have identified a recombination-activating gene (RAG-1) that activates V(D)J recombination in a cell line that normally does not express such activity (Schatz et al., 1989). However, the product of RAG-1 has not been identified, and therefore it is not known whether the activation is by coding a component of V(D)J recombinase itself or by activating other components/factors that carry out V(D)J recombination. The V(D)J recombination system may consist of several enzymes, among which some or all may be lymphoid specific. Terminal deoxynucleotidyltransferase (TdT) is the only well-characterized enzyme that has been implicated in V(D)J recombination where it seems to be responsible for the insertion of a small number of nongermline-encoded nucleotides at the V–D and D–J junctions (Landau et al., 1987). Other activities expected to be associated with the recombinase system are coordinated recognition of RSSs, cleavage between the RSSs and their flanking gene segments, and ligation of the cleaved ends (Hope et al., 1986). A number of factors with heptamer binding (Aguilera et al., 1987), nonamer binding (Halligan & Desiderio, 1987), and endonucleolytic (Hope et al., 1986; Desiderio & Baltimore, 1984) activities have been detected in nuclear extracts from lymphoid cells, but their relationship with V(D)J recombination or with RAG-1 remains unclear. The idea that a single recombinase system assembles different TCR/Ig gene segments and that TdT could be a part of this system prompted us to look for such recombinase activity in various soluble and insoluble subnuclear thymic fractions of 2- to 3-week-old rats. In an attempt to detect recombinase activity in these subnuclear fractions, we have developed an in vitro assay system using an extrachromosomal DNA as the V(D)J recombination substrate. Using this assay system, we have successfully demonstrated that recombinase activity is enriched in the insoluble nuclear matrix fraction of rat thymus gland and is capable of carrying out in vitro V(D)J recombination. Results have also indicated that V(D)J recombination activity associated with the thymic nuclear matrix is lymphoid specific and detectable only during early stages of development. On the other hand, similar preparations from spleen and liver and from the thymus of older rats (>25 weeks) failed to exhibit V(D)J recombination under similar conditions.

Materials and Methods

Deoxyribonucleoside triphosphates (dNTPs), ribonucleoside triphosphates (rNTPs), dithiothreitol (DTT), Nonidet P40 (NP40) synthetic oligomeric DNA, and activated calf thymus DNA were the products of Sigma Chemical Co.; radioactive dNTPs were obtained from the Isotope Division of Bhabha

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[1] Abbreviations: TdT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate; DTT, dithiothreitol; Ig, immunoglobulin; TCR, T-cell receptor; V, D, and J, variable, diversity, and joining segments of Ig or TCR genes; NP40, Nonidet P40; SDS, sodium dodecyl sulfate; Cam, chloramphenicol; AMP, ampicillin; Cam^r, chloramphenicol resistant; AMP^r, ampicillin resistant.

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Atomic Research Centre, Bombay, India. All other reagents were of high-purity grade obtained from SISCO Research Lab, Bombay.

Buffers. Tris-HCl/sucrose buffer contains 5 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, and 0.25 M sucrose. Low-salt buffer (LSB) contains 20 mM Tris-HCl, pH 7.4, 1 mM DTT, 1 mM PMSF, and 0.2 mM $MgCl_2$.

Fractionation of Thymus Nuclei. Thymus glands excised from 2–3-week-old rats (Wistar strain) were immediately transferred to ice-cold Tris-HCl/sucrose buffer. The glands were minced, and nuclei were isolated as described previously (Pandey et al., 1989; Dave et al., 1989). The pure nuclear pellet was resuspended in the same buffer (1.5 mg of DNA/mL) and incubated at 5 °C for 16–20 h. The endogenously digested nuclei were then extracted six times with 5 volumes of LSB, twice with 0.5 M NaCl, and once with 1 and 2 M NaCl, respectively, in the same buffer. Aliquots of soluble and insoluble fractions were withdrawn at each step of extraction and stored at −70 °C.

In Vitro Assay for V(D)J Recombination. V(D)J recombination activity in the soluble and insoluble subnuclear fractions was assayed by use of the pJH200 plasmid as the recombination substrate. A typical reaction mixture for the in vitro V(D)J recombination assay contained 10 mM Tris-HCl, pH 7.4, 1–3 μg of pJH200 DNA, 100 μM each dATP, dGTP, dCTP, and dTTP, 2 mM ATP, 1 mM DTT, 5 μg of bovine serum albumin, 10 mM $MgCl_2$, and an aliquot of subnuclear fraction equivalent to 1–10 μg of protein in a final volume of 100 μL. Reactions were carried out at 37 °C for 30 min and terminated by the addition of EDTA to a final concentration of 20 mM. The reaction mixtures were chilled and centrifuged at 5000*g* for 5 min. Either an aliquot (10 μL) of the supernatant was used directly for the transformation of *Escherichia coli* (HB 101) or the plasmid DNA in the supernatant was recovered by precipitation with alcohol, and 100–300 ng of DNA was then used for the transformation following standard protocol (Sambrook et al., 1989). Approximately 40% of the transformation mix was spread on an LB agar plate containing 100 μg of ampicillin (AMP)/mL of the medium; the remaining 60% was spread on an LB agar plate containing AMP and chloramphenicol (Cam) at a final concentration of 100 and 11 μg/mL, respectively. Plates were incubated at 37 °C for 16–20 h for growth of AMP^r transformants and for 30–36 h for AMP^rCam^r transformants.

Assay for Chloramphenicol Acetyltransferase (cat) Expression in AMP^rCam^r Transformants. Approximately 10% of the doubly resistant colonies was picked from the plates grown separately in LB medium containing only chloramphenicol. Cells were harvested during mid log phase and assayed for *cat* expression as described by Shaw (1975). Almost all of the doubly resistant transformants tested were found to be positive for *cat* expression.

Assay for Nuclease Activity in the Subnuclear Fractions. The level of nuclease activity in the subnuclear fraction was determined as follows: 5 μg of pJH200 DNA was nick translated by use of 10 μCi of [α-^{32}P]dTTP as the labeled dNTP substrate (Sambrook et al., 1989). The product of the nick-translation reaction was ligated by use of T4 DNA ligase and then diluted with cold pJH200 DNA to obtain a specific activity of approximately 10^5 cmp/μg of DNA. The labeled DNA was then used as the substrate for estimation of nuclease activity in the subnuclear fractions. A typical reaction mixture contained 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 100 μg of BSA/mL, 1 μg of ^{32}P-labeled pJH200 DNA, 8 mM $MgCl_2$, and an aliquot of subnuclear fraction equivalent to 5 μg of protein in a total reaction volume of 50 μL. The reaction was carried out at 37 °C for 30 min and terminated by the addition of ice-cold 5% trichloroacetic acid (TCA). TCA-insoluble material was collected on Whatman GF/B glass fiber filters and counted for radioactivity as described previously (Pandey & Modak, 1987). The ^{32}P radioactivity of pJH200 DNA rendered susceptible to acid precipitation was the measure of the nuclease activity in the subnuclear fractions.

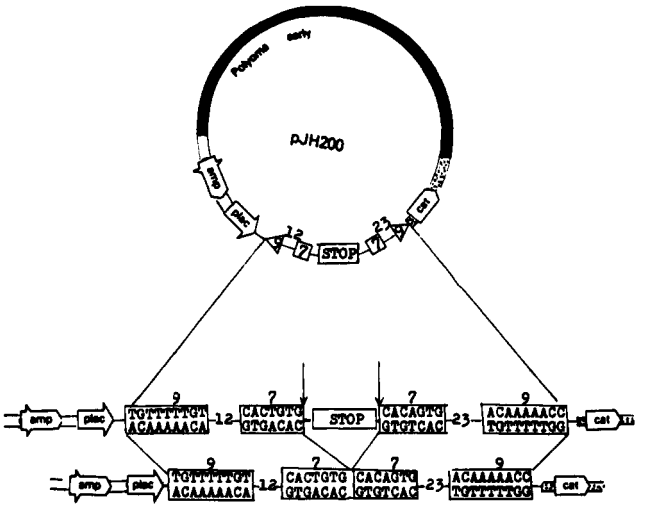

FIGURE 1: Structure of V(D)J recombination substrate. The structural details and construction of plasmid pJH200 having the polyoma early region, pUC13 sequences, and the *cat* gene have been described by Gellert and his colleagues (Lieber et al., 1988; Hesse et al., 1987). It contains heptamer/nonamer immunoglobulin joining signal sequences flanking an *oop* transcription terminator (STOP) of bacteriophage λ present on a 189-bp fragment. The signals are in turn flanked by the *E. coli* lac promoter (plac) on one side and the gene for *cat* on the other. Transcription of the *cat* gene from the lac promoter is blocked by the transcription terminator when the plasmid is in *E. coli*. V(D)J recombination by subnuclear fractions results in a deletion of the terminator followed by fusion of the joining signals, thus allowing *cat* expression when it is introduced into the bacterial host.

In some of the experiments, ^{32}P-labeled calf thymus activated DNA was also used as the substrate for the nuclease assay. The activated DNA was labeled with the use of [α-^{32}P]dNTP substrates in an *E. coli* pol I (Klenow fragment) mediated reaction (Pandey & Modak, 1988). The labeled DNA was purified on a spun column (Sambrook et al., 1989) before use in the nuclease assay.

In Vitro Assay for V(D)J Recombination Activity in Other Organs. In vitro recombination reactions were also carried out with soluble and insoluble subnuclear fractions prepared from liver, kidney, and spleen from 2–3-week-old rats. These organs were found to be devoid of V(D)J recombination activity as judged by the complete absence of AMP^rCam^r transformants even after prolonged incubation.

RESULTS

To assay V(D)J recombination activity in various subnuclear fractions of rat thymus, we have used plasmid pJH200 (a kind gift from Dr. M. Gellert, NIH, Bethesda) as the V(D)J recombination substrate (Lieber et al., 1988; Hesse et al., 1987). This plasmid encodes genes for antibiotic resistance as well as V(D)J recombinational signal sequences flanking a prokaryotic transcription terminator (Figure 1). The signals are in turn flanked by the *E. coli* lac promoter (plac) on one side and a gene for chloramphenicol acetyltransferase (cat) on the other. The bacterial host transformed with this plasmid is resistant to ampicillin (AMP^r) but remains sensitive to chloramphenicol (Cam) due to the presence of a transcription

Table I: V(D)J Recombination Activity in Soluble and Insoluble Subnuclear Fractions of Thymus Nuclei from 2- to 3-Week-Old Rats[a]

fraction	% V(D)J recombination	specific V(D)J recombination activity ($\times 10^{-3}$)
control (with buffer but without fraction)	0	0
nuclei	0	0
soluble subnuclear fractions		
LSB extract	0	0
0.5 M salt extract	0	0
1.0 M salt extract	2.3	0.07
2.0 M salt extract	6.0	2.0
insoluble subnuclear fractions		
LSB insoluble nuclear matrix (NM)	2.4	0.9
0.5 M salt insoluble NM	3.6	2.5
1.0 M salt insoluble NM	32.1	18.5
2.0 M salt insoluble NM	33.3	19.2

[a] The soluble and insoluble subnuclear fractions of thymus nuclei from 2- to 3-week-old rats were prepared and assayed for in vitro V(D)J recombination activity as described in Materials and Methods. A control containing all the reaction reagents except the fraction was also processed similarly. Percent recombination and specific V(D)J recombination activity obtained with each fraction were determined as described in the text; percent recombination activity is determined according to (AMP^rCam^r/AMP^r) × 100, and the specific V(D)J recombination activity is determined from the number of AMP^rCam^r colonies per milligram of protein.

terminator upstream of the *cat* gene. The RSS-directed in vitro recombination of this plasmid causes deletion of intervening DNA containing the terminator sequence. This results in the activation of *cat* gene expression when it is introduced in *E. coli*. The ratio of AMP^rCam^r transformants to AMP^r transformants reflects the fraction of DNA that has undergone V(D)J recombination.

Following endogenous nuclease digestion, the thymic nuclei from 2- to 3-week-old rats were fractionated into soluble and insoluble fractions by stepwise extraction with low-salt buffer (LSB) and with the salt at a concentration of 0.5, 1.0, or 2.0 M in the same buffer (Pandey et al., 1989, 1990). Aliquots of soluble and insoluble fractions obtained at each step of extraction were examined for in vitro V(D)J recombination activity under standard conditions (see Table I and Figure 2) with use of pJH200 as the recombination substrate. After the in vitro recombination reaction, the plasmid DNA was recovered and introduced in *E. coli* and the extent of V(D)J recombination arising from V(D)J recombinase activity was monitored by screening for AMP^rCam^r colonies. The percent recombination was calculated from the ratio of AMP^rCam^r transformants to AMP^r transformants, whereas specific recombinase activity was determined from the number of recombinants (AMP^rCam^r) obtained per milligram of protein in each fraction. The results presented in Table I show that V(D)J recombination activity was detectable in each of the fractions except LSB and 0.5 M salt extracts and that this activity was enriched in the insoluble fractions following successive extraction steps. The soluble fractions extracted with 1 M and 2 M salt exhibited some degree of recombinase activity as judged by the low level of recombination (2–6%). The extent of V(D)J recombination obtained with insoluble subnuclear fractions varied from 2–3% with the LSB and the 0.5 M salt-insoluble nuclear matrices to 32–33% with high-salt-insoluble nuclear matrices. Similarly, specific V(D)J recombination activity varied from 900 to 19 200, the highest being with high-salt-insoluble nuclear matrices.

The level of nuclease activity in soluble and insoluble subnuclear fractions was estimated to ascertain whether failure to detect in vitro V(D)J recombination activity in some of the soluble fractions is due to a high level of nuclease activity in the fraction. The nuclease activity was determined by measuring the extent of degradation of ^{32}P-labeled recombination substrate (pJH200) or calf thymus activated DNA under the standard assay conditions (see Materials and Methods). It was observed that nuclease activity estimated

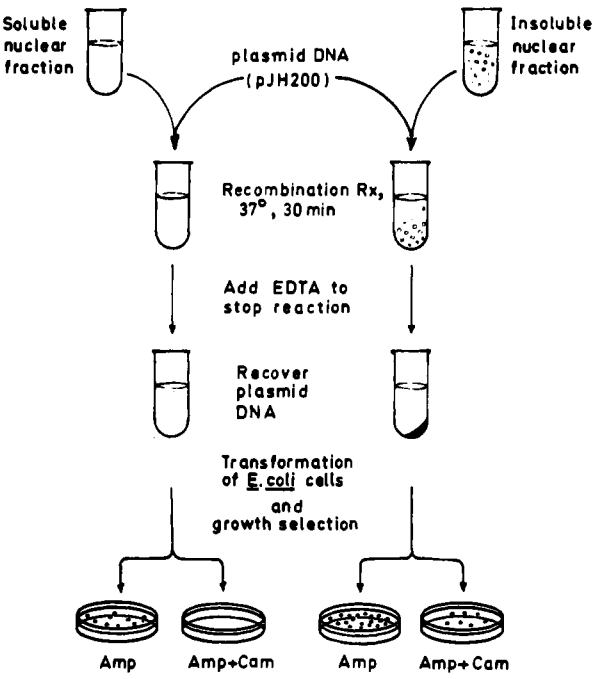

FIGURE 2: Scheme for in vitro V(D)J recombination assay in various subnuclear fractions of thymus nuclei from 2- to 3-week-old rats. An aliquot of the subnuclear fraction equivalent to 1–10 μg of protein and 1–3 μg of pJH200 DNA was incubated in a typical reaction mixture (see Materials and Methods) at 37 °C for 30 min. The reaction was stopped, and the plasmid DNA recovered from the reaction mixture was used for transformation of *E. coli* (HB 101). The input pJH200 encodes both AMP resistance and Cam resistance, but the presence of a terminator sequence downstream of the lac promoter blocks the transcription of the *cat* gene in the bacterial host. In vitro recombination of this plasmid during a 30-min incubation with thymic subnuclear fractions causes deletion of the terminator and thereby restores the expression of the *cat* gene when it is introduced in *E. coli*.

with activated DNA was highest in the LSB extract followed by 0.5, 1.0, and 2 M salt extracts in decreasing order (Table II). The pattern was reversed when recombination substrate pJH200 DNA was used as the substrate for nuclease. Similar results were also obtained with insoluble subnuclear fractions where the level of nuclease activity in LSB and 0.5, 1.0, and 2.0 M salt insoluble nuclear matrices was in decreasing order when assayed with activated DNA and in increasing order with the pJH200 DNA. The observed high or low level of nuclease activity with activated DNA and pJH200 DNA could be

Table II: Distribution of Nuclease Activity in Subnuclear Fractions and Its Effect on the Recovery of Recombination Substrate (pJH200) and on the Efficiency of Transformation and V(D)J Recombination[a]

subnuclear fraction	% of maximal nuclease activity		% recovery of pJH200 DNA after in vitro reaction	AMP^r colonies per μg of recovered pJH200 (×10^{-3})	AMP^{r}Cam^r colonies per μg of recovered pJH200
	pJH200 DNA	activated DNA			
control (without fraction)	0	0	98	6.0	0
soluble subnuclear fractions					
LSB extract	25	100	93	3.6	0
0.5 M salt extract	68	60	81	3.0	0
1.0 M salt extract	85	48	66	2.3	60
2.0 M salt extract	100	5	52	1.5	90
insoluble subnuclear fractions					
LSB insoluble NM	18	95	91	2.5	61
0.5 M salt insoluble NM	24	63	87	2.3	54
1.0 M salt insoluble NM	34	29	78	2.1	669
2.0 M salt insoluble NM	36	21	70	2.0	674

[a] The assay of nuclease activity in the subnuclear fractions was carried out as described in the Materials and Methods with use of either ^{32}P-labeled pJH200 DNA or ^{32}P-labeled activated DNA as the substrate. The maximum ^{32}P radioactivity values of the labeled pJH200 DNA and activated DNA rendered susceptible due to nuclease activity were 2.1×10^4 and 9.6×10^4 cpm/μg of protein in the fraction, respectively. In vitro recombination reaction with each of the fractions was also carried out, and the amount of pJH200 DNA recovered after the reaction was estimated by spectrofluorometric assay (Labarca & Pigen, 1980). The amount of endogenous DNA contributed by the subnuclear fraction was also taken into account to estimate the pJH200 DNA recovery after the recombination reaction. The results are expressed as percent recovery of pJH200 DNA with respect to the DNA recovered from the control without subnuclear fraction. An aliquot of the recovered plasmid DNA (equivalent to 100 ng) from each set of experiments was used for the transformation of *E. coli* cells (HB 101). The results are expressed as the number of singly resistant (AMP^r) and doubly resistant (AMP^{r}Cam^r) colonies obtained per microgram of recovered pJH200 DNA.

related to the ratio of exonucleases and endonucleolytic enzymes in the fractions and shows no distinct relationship with V(D)J recombination activity.

Experiments were also carried out to estimate the recovery of plasmid DNA following the in vitro recombination reaction and the extent of transformation with the recovered plasmid DNA. The recovery of plasmid DNA was found to be lowest with high-salt extracts and also matches well with the level of nuclease activity (with pJH200 DNA) in the other fractions. The number of AMP^r transformants obtained per microgram of recovered DNA showed an inverse relationship with the nuclease activity, suggesting degradation of the recombination substrate (Table II). However, the number of AMP^{r}Cam^r transformants per microgram of recovered DNA varied from 60 with the 1 M salt extract to 674 with the high-salt-insoluble nuclear matrix. Under similar conditions, the doubly resistant colonies were completely absent in the control, although the extent of transformation, as judged by the total AMP^r colonies, was highest with the plasmid DNA recovered from the control (Table II).

Our earlier observations have indicated that high-salt extraction of endogenously digested nuclei of the thymus gland from 2- to 3-week-old rats removed a major portion of nuclear TdT, though a small but significant amount of the enzyme remains tightly bound to the residual nuclear matrix (Pandey et al., 1989, 1990). For the sake of simplicity, we have designated TdT in soluble extract and in the residual nuclear matrix as free and matrix-bound TdT, respectively. On the basis of the response of free and bound TdT to ATP, a potent inhibitor of this enzyme with a $K_i = 5$ μM, we have suggested that ATP-resistant matrix-bound TdT could be the functionally organized form of the enzyme, whereas free TdT may be rendered nonfunctional under strong negative regulation of ATP in prelymphocytes (Dave et al., 1989). Hence, it was of interest to ascertain whether any correlation between TdT and V(D)J recombination activities exists in subnuclear fractions of thymus glands excised from 2- to 3-week-old rats. Table III summarizes the results where specific activity of V(D)J recombination is compared with that of TdT in soluble and insoluble subnuclear fractions. Results indicated a remarkable correlation between specific TdT activity and specific V(D)J recombination, which is consistently higher in the high-salt-insoluble nuclear matrix. Further observations that nuclear matrices of liver and spleen from 2- to 3-week-old rats and thymus nuclear matrix from older rats (25 weeks) were completely devoid of both activities (data not shown) suggest that association of these activities with the nuclear matrix is not only lymphoid specific but also restricted to early stages of development.

Table III: Correlation between Specific Activities of V(D)J Recombination and Terminal Deoxynucleotidyl Transferase (TdT) in the Insoluble Subnuclear Fractions of Thymus Nuclei[a]

subnuclear fraction	specific TdT activity	specific V(D)J recombination (×10^{-3})
LSB insoluble NM	7.0	0.9
0.5 M salt insoluble NM	12.0	2.5
1.0 M salt insoluble NM	28.0	18.5
2.0 M salt insoluble NM	48.0	19.2

[a] Aliquots of insoluble subnuclear fractions obtained at each step of extraction of endogenously digested nuclei were assayed for TdT activity as described before by use of [^{3}H]dGTP as the substrate and activated DNA as the initiator (Pandey et al., 1989). Specific activity of the enzyme was expressed as picomoles of dGMP incorporated per milligram of protein under standard assay conditions. Values for specific V(D)J recombination activity in the insoluble fractions were taken from Table I.

Discussion

High-salt-resistant nuclear matrix is a residual nuclear structure remaining after extraction of the bulk of chromatin and soluble components from isolated nuclei (Berezney & Coffey, 1974). A number of recent studies have shown that replicative DNA polymerase and other related enzymes involved in replication are tightly associated with the nuclear matrix, suggesting it to be an important intranuclear site for DNA replication (Smith & Berezney, 1982; Tubo & Berezney, 1987). The association of V(D)J recombination activity observed in the nuclear matrix of thymus glands suggests that the nuclear matrix could also be the site for in vivo V(D)J recombination. Although DNA replication and V(D)J recombination are distinct processes, they share certain common features; e.g., two processes involve site-specific multienzyme

participation that includes recognition of specific sequences for initiation of their functions, removal of mismatching or intervening DNA, and ligation of replicated or rearranged DNA fragments. Both processes would require the concerted action of several enzymes or proteins in close proximity with one another. The nuclear matrix could provide an ideal site for their organization. We therefore postulate that, similar to the DNA replicational complex, various components/factors associated with V(D)J recombination are also integrated in the nuclear matrix in the form of a macromolecular recombinational complex or recombinase system and that the matrix-bound TdT could also be a part of this system. Our recent observation that matrix-bound TdT exists as a 23S multienzyme complex containing TdT, DNA ligase, exonuclease, and several unidentified components also supports this contention (Pandey et al., 1990). However, it has not been possible to demonstrate in vitro V(D)J recombination with the sucrose-gradient-purified 23S multienzyme complex, although this activity is clearly demonstrated in the matrix extract. We presume that certain component(s) or factor(s) essential for the recombination may have been dissociated and sedimented at different positions of the gradient during the purification of the soluble complex by prolonged centrifugation. The ability of individual sucrose-density-gradient fractions of the matrix extract to carry out in vitro V(D)J recombination with or without the 23S fraction is being examined, and these results will be published elsewhere. Further studies on this recombinase system in the matrix-bound as well as in the solubilized state may provide a valuable approach for investigating the organization, functional properties, and cell-phase-dependent assembly and disassembly of the recombinational apparatus. Although it remains to be determined whether TdT and the associated recombinational apparatus are actually bound to the nuclear matrix in vivo, the association of V(D)J recombination activity with the isolated nuclear matrix provides a potentially important in vitro system for studying the organization of the putative recombinase system and the possible biochemical mechanism of V(D)J recombination of TCR/Ig gene segments in prelymphocytes.

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