V(D)J Recombination on Minichromosomes Is Not Affected by Transcription*

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It has been shown previously by others that transcription is temporally correlated with the onset of V(D)J recombination at the endogenous antigen receptor loci. We have been interested in determining whether this temporal correlation indicates a causal connection between these two processes. We have compared V(D)J recombination minichromosome substrates that have transcripts running through the recombination zone with substrates that do not in a transient transfection assay. In this system, the substrates acquire a minichromosome conformation within the first several hours after transfection. We find that the substrates recombine equally well over a 100-fold range in transcriptional variation. In additional studies, we have taken substrates that have low levels of transcription and inhibited transcription further by methylating the substrate DNA or by treating the cells with a general transcription inhibitor (a-amanitin). Although these treatments decrease the level of expression an additional 10-100-fold, there is still no observable effect on V(D)J recombination.

Based on these results, we conclude that transcription is not necessary for the V(D)J reaction mechanism and does not alter substrate structure at the DNA level or at the simplest levels of chromatin structure in a way that affects the reaction.

In lymphoid differentiation, not all of the seven T-cell receptor and immunoglobulin gene loci are available for rearrangement at the same time. A fundamental question about V(D)J recombination concerns how the enzyme system is targeted to chromosomal loci in a developmentally and temporally controlled manner. Various hypotheses have been proposed to explain this locus control of rearrangement. These have included primary roles for transcription, replication, demethylation, and chromatin structure. In studies of the endogenous loci or of transgenes, it has been difficult or impossible to dissect these parameters apart. Rearrangement at the endogenous loci is temporally correlated with an increase in endonuclease sensitivity, an onset of transcription, and demethylation (1-8). But the causal relationship of these relative to recombination is still unclear.

Transcription has received particular attention as a possible prerequisite for recombination. The long-standing observation of a temporal linkage between recombination and transcription has been strengthened with functional studies in which B cell-specific transcriptional regulatory sequences have been placed around T cell receptor segments in integrated constructs or in transgenes. In these studies, the segments acquire the recombination propensity corresponding to the lineage specificity of the transcriptional sequences (2, 9). On this basis, a causal role for transcription has been considered even more likely. However, other studies examining integrated substrates that either do or do not contain promoter elements have not found as strong a role for transcription per se in the targeting process (10). In recent detailed examinations of the endogenous loci, establishment of a causal connection between transcription and recombination has also been complicated. Some studies using the RNA polymerase chain reaction have indicated an extremely tight linkage between the temporal onset of transcription and recombination (6). However, at other loci, transcriptional analysis in one direction for one of the several D segments indicated no detectable transcription prior to D to Jβ rearrangement (11). Hence, there has been a persisting uncertainty about the precise role that transcription might play, if any, in directing the recombination reaction.

The ways in which transcription and recombination might be causally related include (a) mechanistic coupling and (b) requisite effects of transcription on substrate DNA structure (e.g. superhelical tension or stabilization of single-stranded regions) or on substrate chromatin structure. Alternatively, the association between transcription and recombination could simply be a temporal one and due to the necessity for a chromatin change before DNA-binding proteins for both transcription and recombination can have access to their respective recognition sites. Some of these relationships are readily testable using plasmid minichromosomes. This approach has the advantages that recombination can be quantitated and that transcription can be varied by removal of promoter and enhancer elements, by methylation, or by addition of general transcription inhibitors.

MATERIALS AND METHODS

Plasmids—All plasmids used here have been described previously (12-14). Salient features relevant to this study are pointed out here. The plasmid pML200 is 6.2 kbp in length and is identical to pH200 except for the removal of an Spel restriction fragment from the T antigen coding sequence region (polyoma positions 780-1977, Soeda et al.)

The abbreviations used are: kb, kilobase pair(s); bp, base pair(s); SDS, sodium dodecyl sulfate; A, ampicillin; AC, ampicillin-chloramphenicol; DA, transformants arising on ampicillin plates from DNA that has been treated with DpnI; EGTA, (ethylenebis(oxyethylendinitrilo))tetraacetic acid.

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Transcription Does Not Affect V(D)J Recombination

cell line, 1-8, in 10% fetal calf serum, 90% RPMI 1640, 50 mM 2-mercaptoethanol, 100 units of penicillin/streptomycin (9, 10). We transfected plasmids into the cell line by a hypotonic treatment of the DEAE-dextran method (13). We recovered plasmid DNA (48 h after transfection unless otherwise specified) using a rapid alkaline lysis protocol (17).

V(D)J Recombination Assay—as described in detail previously, we detected recombinants from the V(D)J recombination assay by a bacterial transfection assay (co-transfection with luciferase expression vectors). Recombinants were detected as described (18). We distinguished repaired plasmid DNA from DNA entered but not repaired by cleaving unrepaired plasmid DNA with a methylation-sensitive restriction enzyme, HpaII (14). We distinguished repaired plasmid DNA from DNA entered but not repaired by cleaving unrepaired plasmid DNA with a methylation-sensitive restriction enzyme, HpaII (14). We distinguished repaired plasmid DNA from DNA entered but not repaired by cleaving unrepaired plasmid DNA with a methylation-sensitive restriction enzyme, HpaII (14). We distinguished repaired plasmid DNA from DNA entered but not repaired by cleaving unrepaired plasmid DNA with a methylation-sensitive restriction enzyme, HpaII (14). 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We distinguished repaired plasmid DNA from DNA entered but not repaired by cleaving unrepaired plasmid DNA with a methylation-sensitive restriction enzyme, HpaII (14). We distinguished repaired plasmid DNA from DNA entered but not replicated and, therefore, retain the dam methylation. We designate treatment with DpnI as a D. Hence, transformants arising on ampicillin plates from DNA that has been treated with DpnI are designated DA. The percentage of a population of recovered plasmids that has replicated is 100 × (DA/A). When a substrate has been co-transfected with pHJ104, a plasmid that supplies T antigen in trans, pHJ104 is eliminated from the calculation by digestion with NarI, SmaI, or SpAl.

Assays for Entry of Non-replicating Plasmids into Eukaryotic Cells—When plasmids bearing a polyoma origin of replication are introduced into eukaryotic cells, replication of the plasmid DNA enters the eukaryotic cells. Two alternatives were used in different experiments here. (i) Co-transfection with an expression vector can be used to estimate entry of the substrate plasmids into the cells. (ii) The substrate entry can be followed directly by monitoring DNA repair of minor, intentionally placed damage on the substrate DNA. Each method has different advantages.

The expression vector used here, pRSVL, transcribes the luciferase gene. Its expression is assayed as follows. A sample of 1.5 × 10⁶ transformed cells is harvested at the indicated time after transfection and assayed in a luminometer (Analytical Luminescence Laboratories, Moonlight Beach, England). In the DNA analysis, pRSVL is eliminated by MluI restriction digestion.

An alternative to co-transfection with luciferase expression vectors is to follow DNA repair of minor, intentionally placed damage. Recently, we described an assay in which we substituted doxycycline for 10-20% of the thymidine residues in the plasmid DNA. The plasmids which enter the lymphoid cells are repaired efficiently in vivo by the eukaryotic uracil DNA repair system. Upon plasmid recovery 48 h after transfection, we distinguish repaired (entered) from unrepaired (not entered) plasmids by cleaving unrepaired plasmid molecules with uracil DNA glycosylase to remove the uracil residues, followed by an alkaline lysis step, followed, as before, by colchicine and heat treatment. Heat treatments renders the cleaved molecules non-transformable in the bacterial transformation assay.
Experimental Strategy—For our studies we have used an extrachromosomal substrate assay system described previously (14, 17). In this system, the heptamer-nonamer recombination signals have been removed from their chromosomal context and placed on plasmids. The recombination process can be rapidly assayed with sensitivity and quantitation as follows. The recombination signals are positioned around a prokaryotic transcription terminator. The antibiotic resistance marker, chloramphenicol acetyl transferase (cat), is positioned downstream of this complex, and a prokaryotic promoter is upstream. Plasmids that have been recombined by the lymphoid V(D)J recombinase have deleted the prokaryotic transcription terminator that blocks transcription into the cat gene. Hence, recombinant plasmids confer chloramphenicol resistance upon host bacteria. Both substrate and product plasmids confer ampicillin (A) resistance. Recombinants are detected by recovering the extrachromosomal DNA from lymphoid cells, transforming E. coli, and plating transformants onto AC double-selection plates.

Transfection of the substrates into lymphoid cells can be normalized in any of three ways. All of the V(D)J recombination substrates used in this study except pH100 and pML20 are carry the polynoma origin of replication, but none carry a complete polynoma large T gene. In the cases where a large T expression vector is co-transfected with a polynoma origin bearing substrate, the large T expression supports the replication of the substrate and recombination determinations can be normalized for plasmid replication (12, 14) (see “Materials and Methods”). In cases where we have assessed recombination on plasmids under non-replicating circumstances (no polynoma origin or no large T), we have either normalized for transfection efficiency using (a) DNA repair (12) or (b) luciferase expression from a co-transfected expression vector (see “Materials and Methods”).

Recombination as a Function of Transcription on Replicating Substrates—Our initial studies of recombination as a function of transcription are under circumstances where the substrates replicate. pML20 contains the early region of polynoma with some modifications. The early region consists of the polynoma late promoter, the polynoma early promoter and T antigen genes, and the polynoma enhancer between them. The two promoters direct transcripts in opposite directions around these 4–5-kb plasmids. A 1.3-kb portion of the T antigen gene sequences has been removed (12) so that large T must be supplied in trans from another plasmid in order for pML20 to replicate. However, the large T promoter and the initial portion of the T antigen gene have been left unaltered. In contrast, pML201 has only the polynoma late promoter and enhancer and does not contain the early promoter. Because pML201 has only one promoter, we expected the transcription on pML201 to be reduced compared with pML20. In addition, a eukaryotic transcription terminator (16) has been positioned to block transcription arising from the remaining late promoter in pML201 (Fig. 1 and “Materials and Methods”). Therefore, the total transcription on the plasmid and through the recombination zone was likely to be substantially lower on pML201 than for pML200.

For our initial studies, we transfected pre-B lymphoid cells with either pML201 or pML200, each along with pH104, a T antigen expression vector. We harvested samples for recombination determinations and RNA analysis 48 h after transfection. We were interested in the level of transcription on either strand through the recombination region and not only from these two promoters, but also from any adventitious transcription start sites on the plasmid. Therefore, we hybridized immobilized total RNA with radiolabeled DNA probes corresponding to the 350-bp recombination region of the plasmid (Fig. 1). We normalized for RNA recovery by reprobing these blots for a-actin, and we normalized for template entry (transfection efficiency) by using replication as a marker. Transcripts arising from transcription through the recombination region are reduced approximately 100-fold on pML201 as compared with pML200 (Fig. 2). Despite this decrease in transcription, recombination is not significantly affected (Table I).

Recombination as a Function of Transcription on Non-replicating Substrates—The studies above suggested that

![Fig. 1. Transcription and recombination on the substrates.](image-url)
there were no large effects of transcription on recombination. We were concerned that any structural or topological linkage between recombination and transcription, in some way, might be masked by replication. Therefore, we conducted a larger study similar to the one above, but under circumstances in which the plasmids do not replicate. Five substrates were used in this study (Table II). As mentioned, pML200 bears two viral promoters (early and late) and a viral enhancer; pJH229 has only the late viral promoter and has the viral enhancer; pML201 is identical to pJH229, but with a transcription terminator positioned to block transcription from the late promoter; pJH100 bears no eukaryotic promoters, but does have the Ig κ intron enhancer; pML98 has neither promoters nor enhancers (Fig. 1). None of these substrates is capable of autonomously reproducing in the eukaryotic cells.

We co-transfected each of these substrates into the pre-B cell line 1-8 with a luciferase expression vector, pRSVL. At 48 h after transfection, we harvested three types of samples from the transfections: DNA for recombination analysis, RNA for the assay of transcripts corresponding to the recombination zone, and cytoplasmic cell lysates for luciferase analysis. Relative levels of transcripts corresponding to the recombination zone of pML200, pJH229, pML201, and pJH100 were found to vary over a 100-fold range (Table II). (pML98 was not analyzed for transcription. Because this plasmid is identical to pJH100 except for the deletion of an enhancer, we anticipate that this plasmid would also have undetectable levels of transcripts through the region.) The relative difference in transcription between pML200 and pML201 remained similar in this analysis using non-replicating plasmids (Table II) as compared with the relative difference for the same substrates examined under replicating conditions (Table I). Likewise the relative ratios of transcription of the other plasmids to one another remained the same in multiple analyses. Examination of the recombination efficiency of these substrates showed that there were no significant differences between them (Table II and Fig. 1). Analysis of the signal joint for precision of recombination demonstrates that approximately 90% have no nucleotide loss or addition and that 10% have non-germline addition (not shown). These signal joint features do not change with the transcriptional activity of the substrate. We infer that variation of transcription over a wide range has no effect on V(D)J recombination.

**Regional Inhibition of Transcription Also Does Not Affect V(D)J Recombination**—Although our studies indicated that V(D)J recombination on these substrates was not influenced by transcription through the recombination zone, we were concerned about two possibilities. One possibility is that these processes are linked, but over a range of transcription that is even lower than our least transcriptionally active plasmid. That is, very low levels of transcription might be sufficient to activate the process. A second possibility is that transcription through the recombination zone is not important, but that transcription in the adjacent DNA is adequate. (Our measurements of transcription through the recombination zone

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**Table I**

Recombination as a function of transcription on replicating plasmids

Pre-B cells were transfected with pJH104 and either pML200 or pML201. At 48 h, samples for DNA and RNA analysis were taken. RNA samples were probed for the level of transcripts through the recombination sites (rec sites) or for cellular actin. DA (DpmIAMP') is the number of replicated templates recovered in 1/80 of the DNA sample from the transfected cells. The level of transcripts from the recombination zone normalized for cellular RNA and for RNA entry (DA) is given as sites/actin × DA). The background transcript level through the recombination zone (rec sites) was established by mock transfection of the lymphoid cells, harvest, and hybridization; the level of hybridization was undetectable and would have been readily detectable if it had been 2% of the pML200 signal. As shown in this experiment, the value of DA is usually somewhat lower for pML200 than pML201. This may be due to the generation of a truncated large T protein by pML200 that may inhibit the replicative increase of the template number. This lower replication rate does not affect the recombination activity because recombination is normalized for replication. The recombination activity level, R, is calculated as DAC/DA.

<table>
<thead>
<tr>
<th>Rec sites</th>
<th>Actin</th>
<th>Rec sites/actin</th>
<th>Template no. (DA)</th>
<th>Normalized transcript level (rec sites × 10^5) (/actin × DA)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>pML200</td>
<td>100</td>
<td>79</td>
<td>1.3</td>
<td>700</td>
<td>180</td>
</tr>
<tr>
<td>pML201</td>
<td>11</td>
<td>100</td>
<td>0.11</td>
<td>8900</td>
<td>1.2</td>
</tr>
</tbody>
</table>

**Table II**

Recombination as a function of transcription on non-replicating plasmids

Pre-B cells were transfected with pRSVL and one of the following plasmids listed in the left column. At 48 h after transfection, samples were taken for RNA, DNA, and luciferase analyses. The ratio (rec sites/actin × Luc) represents the level of transcripts through the recombination zone. As in Table I, the hybridization signal corresponding to pJH100 was undetectable; the designation, <0.7, indicates the signal is below this lower limit of detection. The recombination activity level is calculated as AC/(A × Luc).

<table>
<thead>
<tr>
<th>Rec sites</th>
<th>Actin</th>
<th>(rec sites × 10^5)/actin</th>
<th>Transfection efficiency (Luc)</th>
<th>Normalized transcript level (rec sites × 10^5) (/actin × Luc)</th>
<th>R (× 10^5)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pML200</td>
<td>100</td>
<td>59</td>
<td>169</td>
<td>815</td>
<td>207</td>
</tr>
<tr>
<td>pJH229</td>
<td>5.9</td>
<td>68</td>
<td>9.2</td>
<td>1063</td>
<td>8.7</td>
</tr>
<tr>
<td>pML201</td>
<td>4.3</td>
<td>100</td>
<td>4.3</td>
<td>1067</td>
<td>4.0</td>
</tr>
<tr>
<td>pJH100</td>
<td>&lt;0.7</td>
<td>98</td>
<td>&lt;0.7</td>
<td>1453</td>
<td>&lt;0.7</td>
</tr>
<tr>
<td>pML98</td>
<td>2021</td>
<td></td>
<td>2021</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
constitute 6-8% of the region downstream of the known eukaryotic promoters on the substrates.) In order to test these possibilities, we co-transfected the pre-B cell line 1-8 with the least transcriptionally active plasmids, pML98 or pJH100, and the luciferase expression vector, pRSVL. The transfected population was then divided at 25 h after transfection into three groups. One group was untreated, and the remaining two groups were treated with α-amanitin at concentrations of 9.2 and 19.6 μM. At 50 h after transfection, DNA and luciferase analyses were done. The results for pML98 are shown in Table III. (Very similar results with pJH100 lead to the same conclusion.) We find that cellular transcription reflected by the expression of the co-transfected luciferase vector can be inhibited more than 10-fold. Hence, any undetectably low level of transcription through or in the vicinity of the recombination zone is also likely to be similarly inhibited. Yet no drop in V(D)J recombination is observable.

Although the previous experiment extended our findings that transcription did not affect V(D)J recombination, we wanted to repeat this kind of analysis without the use of a transcription inhibitor like α-amanitin. Therefore, we chose to inhibit transcription on the substrate by CpG methylation.

In other studies, we (13) and others (23) have shown that transiently transfected CpG methylated plasmids do not transcribe or express genes. For studies here, we used the substrates pJH229 and pCLH4 and the expression vector pRSVL. We methylated each of these plasmids at their CpG residues using the CpG methylase, SssI. Complete methylation was documented by subjecting the methylated plasmids and control unmethylated plasmids to digestion with the methylation-sensitive enzyme, HpaII. The methylated plasmids showed no digestion with HpaII, whereas the nonmethylated plasmids showed complete digestion.

We used CpG methylation inhibition of transcription in the following experiment. We transfected pre-B cells with the methylated or nonmethylated versions of pRSVL. At 48 h post-transfection, we divided each transfection sample in two. Half was harvested for analysis of luciferase expression. The other half was harvested to analyze for pRSVL entry into the cells. This was done by harvesting nuclei from the transfected cells, extracting total nuclear DNA, and then fractioning the DNA on an agarose gel, Southern blotting, and probing for the plasmid. We determined the relative amounts of plasmid DNA on an agarose gel, Southern blotting, and probing for the plasmid. We determined the relative amounts of plasmid DNA entry by densitometric analysis. We expressed the results as luciferase activity (minus background) divided by DNA entry (Table IV). This ratio ranged from 0 to 15 (mean, 4.2) for the methylated luciferase vector transfections. In contrast, the control (nonmethylated) plasmids yielded ratios ranging from 173 to 1030 (mean, 510). These observations indicate that CpG methylation inhibits transcription by approximately 100-fold. To determine the effect of CpG methylation on V(D)J recombination, we transfected pre-B cells with the methylated or nonmethylated versions of the substrates pCLH4 or pJH229. We find that V(D)J recombination is not affected (13).

The direction of transcription relative to the orientation of the signals has also been varied in this study. Transcription can occur from the 12-signal toward the 23-signal as in pJH229, or it can occur from the 23-signal toward the 12-signal as in pCLH4. Reduction of transcription did not affect recombination for either substrate. Therefore, it is unlikely that we have missed a role for transcription because of failure to examine one orientation or the other.

The Chromatin Status of Minichromosome Substrates—The above studies indicate that it is very unlikely that active transcription has a mechanistic or DNA structural effect on V(D)J recombination. The previously observed temporal association of transcription with V(D)J recombination (1) might be due, however, to a causal effect of transcription on chromatin structure. In considering this latter possibility, we were interested in evaluating the extent to which our extrachromosomal substrates acquire a chromatin structure upon entry into the pre-B cells. We did two types of analysis to examine the chromatin structure of the substrates. In one analysis, we transfected pre-B cells with the substrate, pJH229, and with a large T expression vector. Nuclei were harvested 48 h after transfection and digested with micrococcal nuclease to determine whether a protection pattern was present in accord with the presence of phased nucleosomes with a periodic spacing (see "Materials and Methods"). Indirect end-labeling analysis of digested DNA indicated that nucleosomes were present in a predominantly phased pattern (Fig. 3). The region of the substrate analyzed by this method was the 3.2 kb including and surrounding the 0.35-kb recombination zone. Except perhaps for the 0.4-kb polya region (24), we believe that this 3.2-kb region is representative of the rest of the 4.6-kb plasmid. The results below confirm this.

In a second type of chromatin analysis, we transfected pre-B lymphoid cells with the substrate pCLH4 under either replicating or non-replicating conditions (i.e. with or without transfection with a large T expression vector). The restriction endonuclease resistance of the substrates was then analyzed by harvesting nuclei from the transfected cells at various

### Table III

Lack of effect of transcriptional inhibition on V(D)J recombination

The plasmid pML98 was used to transfact the pre-B cell line (1-8). α-Amanitin was added at 25 h after transfection of pre-B cells with the substrate pML98 and was not washed out until the cells were harvested at 50 h. Recovered DNA was digested with the restriction enzyme, MluI, prior to bacterial transformation to eliminate the luciferase expression vector as a significant source of ampicillin transformants. The recombination activity level, R, is expressed as recombinants divided by total plasmid (AC/A) readings. DNA structural effect on transfection of the cell population. Background has been subtracted already. Values are the means of readings from three replicate lymphocyte transfections.

<table>
<thead>
<tr>
<th>α-Amanitin</th>
<th>Luc (AC × 10^4/A)</th>
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<tbody>
<tr>
<td>0</td>
<td>1194</td>
</tr>
<tr>
<td>9.2</td>
<td>594</td>
</tr>
<tr>
<td>19.6</td>
<td>99</td>
</tr>
</tbody>
</table>

### Table IV

Levels of expression from plasmids with and without CpG methylation

The luciferase expression plasmid, pRSVL, was methylated or not and transfected into pre-B cells (1-8). After 48 h, two samples were harvested from each of the transfections; one sample was analyzed for luciferase expression. The value is expressed in relative light units minus background. Nuclei were harvested from the other sample from each transfection and analyzed by Southern blot for the amount of pRSVL. The relative template (pRSVL) levels are expressed as densitometric absorbance. The normalized expression is the ratio of luciferase divided by template level.

<table>
<thead>
<tr>
<th></th>
<th>Template normalization</th>
<th>Normalized expression</th>
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<tbody>
<tr>
<td>pRSVL-CpG</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>1.2</td>
</tr>
<tr>
<td>pRSVL</td>
<td>1</td>
<td>814</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>544</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>278</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>690</td>
</tr>
</tbody>
</table>
times after transfection. We found that endonuclease resistance increases markedly by 5 h after transfection and remains high regardless of whether or not the substrates replicate (15). This global chromatin structural analysis indicates that all of the substrates that enter the cells acquire a protected configuration. Hence, there is no unchromatinized sub-population in the isolated nuclei of transfected cells. Moreover, the level of global endonuclease protection that we see is likely to correspond to the phased regional nucleosomal pattern observed in the micrococcal nuclease analysis. Any restriction endonuclease-resistant plasmids that bore unphased nucleosomes would have given rise to a smear obscuring the plasmids giving rise to a phased pattern in Fig. 3. This does not occur. Therefore, we conclude that these substrates acquire a level of chromatin structure. The data are most consistent with a 10-nm fiber chromatin structure, commonly referred to as a beads-on-a-string configuration.

**DISCUSSION**

The striking aspect of these results is that large (1000-fold) reductions in apparent transcription do not affect V(D)J recombination. These studies weigh against proposals for transcriptional effects on V(D)J recombination. First, the results indicate that transcription does not affect the mechanism of V(D)J recombination. Second, they indicate that transcription does not alter DNA structure in a manner that affects V(D)J recombination. Third, there is no effect of transcription on recombination via a requisite alteration in chromatin structure to the extent that these substrates acquire such structure.

Our studies are relatively less complicated by the problem of subpopulations that often confounds the analysis of V(D)J recombination. It is frequently difficult to dissect two parameters because subpopulations of substrates may separately manifest the two different features. For example, transcription might arise from one subpopulation and recombination from another. This is a particular problem when drug selections are imposed on the transfected cells. It is difficult to imagine any kind of heterogeneity in our studies that would account for a failure to see a relationship between transcription and recombination. Because the substrates are not integrated, neither transcription nor recombination can be influenced by chromosomal context.

We have examined recombination over a relatively wide range of transcription. Yet we are mindful that these two processes could be coupled at a level of transcription below our range of analysis. This is a possibility because we have assumed that the steady-state levels of accumulated transcript reflect the rate of transcription, and this assumption is not always true. The transient assay system allows us to quantitate recombination, but measurements of transcription rate in transient transfections are not reliable. This means that we must rely on steady-state transcript levels as a reflection of transcription rate. (The only alternative is to use genomically integrated substrates in which transcription rate measurements are possible but recombination quantitation is not.) Despite the use of steady-state analysis, we believe that our studies are a valid analysis of the relationship between transcription and recombination over a considerable range. Substrates with already low levels of transcription were methylated, resulting in an estimated 100-fold decrease in transcription. There was no effect on recombination. Furthermore, α-amanitin inhibition of transcription on a substrate with no eukaryotic promoters or enhancers has no effect on recombination compared with a substrate with uninhibited viral transcription elements.

In relating the observations in the current study to the events involved in activation of the endogenous loci for V(D)J recombination, it is important to note that higher order chromatin structure is not represented in this analysis. The association between transcription and recombination could still be causal if transcription changed the higher order chromatin structure in a way that was essential for the activation of a locus. How do the studies with minichromosomes described here relate to the large body of literature that indicates that transcription and antigen receptor locus rearrangement are temporally correlated? In the recent study by Ferrier et al. (7), the rearrangement of V to DJ segments was lineage-specific according to the lineage specificity of the promoter. An alternative to supposing that transcription is responsible, however, would be that transcription factors or other specific proteins binding in the promoter region may alter chromatin structure in a way that is important for recombination.

Two other studies have recently questioned a causal linkage between transcription and recombination. In one study, integrated substrates bearing or not bearing a promoter showed equally high recombination (10). In another, activation of D to J\(_H\) joining occurred in association with transcription nearby, but not through the recombining segments (11). Both of these studies indicated that the association of transcription with recombination may not be causal. Rather, the binding of specific proteins in the region, such as at the enhancer, was suggested as a possible alternative prerequisite event. Such proteins might alter chromatin structure or affect the likelihood of the recombinase to bind in this region.

In a separate study, we have found that CpG methylation has a 100-fold inhibitory effect on V(D)J recombination by affecting the chromatin accessibility of the minichromosomes (13). Based on our findings of a strong quantitative effect of CpG methylation on V(D)J recombination, we think that methylation may be one of the critical parameters controlling locus activation for antigen receptor gene rearrangement. For many years, it has been recognized that hypomethylation and rearrangement also share a temporal association (4, 8). In our studies, the inaccessible chromatin structure specified by
methylation requires at least one round of DNA replication (13). Maintenance methylation of these sites is very efficient. Locus activation for rearrangement may require some targeted removal of CpG methylation. We are currently investigating a variety of possible causes for a local interference with the maintenance of the methylated, inaccessible state. The binding of transcription factors to the region could still play a role in changing a higher order chromatin structure to an accessible state. However, this and other potential indirect roles do not include transcription in the reaction mechanism or in the configuring of substrate structure, and our ability to rule out transcription at these simpler levels is important in continuing efforts to detect V(D)J recombination in cell-free biochemical systems.

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