Vol. 181, No. 1, 1991
November 27, 1991

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 95-99

THYMIC NUCLEAR MATRIX ASSOCIATED ACTIVITY IS NOT V(D)J RECOMBINASE[+]

V. N. Pandey[1], V. P. Dave[1], S. B. Amrute[2], K. Rosenbach[2] and M. J. Modak[2*]

[1]Biochemistry Division, Bhabha Atomic Research Centre, Bombay 400 085, India

[2]Department of Biochemistry and Molecular Biology, New Jersey Medical School, University of Medicine and Dentistry of New Jersey, New Jersey 07103-2714

Received October 1, 1991

It was previously reported that nuclear matrix isolated from young rat thymus contained an activity that supported V(D)J recombination at a high efficiency (Dave et al., BIOCHEMISTRY 30: 4763-4767, 1991). A similar type of activity is also detected in the matrix prepared from fetal calf thymus. However, restriction enzyme mapping analyses of the recombined product clearly suggest that the double antibiotic resistance exhibited by the matrix treated plasmid substrate is not a consequence of V(D)J signal sequence recombination.

V(D)J recombination is a site specific recombination that occurs in vertebrates during the process of immunoglobulin gene as well as T cell receptor gene assembly (1-3). A detailed structural analyses of the gene sequences in the embryonic and somatic life of the organism has provided some understanding of the underlying process although the biochemical mechanisms are largely unclear. The heptamer nonamer sequences separated by 12 or 23 base pair spacers appears to be the basic recognition signal sequences for the process of V(D)J gene segment joining. A number of plasmid resistant DNA substrates that contain these

[+]Supported in part by a grant from the National Science Foundation (DMB-8715829) to M. J. M.

*Address correspondence to this author.

Abbreviations used: TdT, terminal deoxynucleotidyltransferase; dNTP, deoxynucleoside triphosphate; Ig, immunoglobulin; TCR, T-cell receptor; V, D, and J, variable, diversity, and joining segments of Ig or TCR genes; Cam, chloramphenicaol; Amp, ampicillin; Cam^r, chloramphenicol resistant; Amp^r, ampicillin resistant.

0006-291X/91 $1.50

recognition sequences have been constructed and successfully used to assess the recombination activity in lymphoid cells (2, 4). The most convenient of these substrates is a plasmid pJH 200 (4-5). This plasmid contains a prokaryotic transcription terminator, sandwiched between the recombination signal sequences. At either end of these signal sequences are ampicillin (Amp) and chloroamphenicol (Cam) resistance genes. Upon successful recombination, the transcription terminator (200 bases long) is removed and both Amp and Cam resistance genes are expressed, resulting in the appearance of a doubly resistant mutant. In the absence of recombination, only the amicillin gene is expressed. It was this assay that indicated the presence of V(D)J recombination activity with rat thymic nuclear matrix. In order to define this system further, we searched for and found fetal calf thymus as an alternative source of thymic cells which contained similar activity. However, when recombinant plasmids, obtained from cells grown on Amp + Cam plates, were analysed by 3 different restriction enzymes and screened for the presence of a unique fragment, none was found. The restriction mapping patterns of parent plasmid and the recombinant plasmids were indistinguishable, suggesting that a mechanism other than V(D)J recombination is responsible for the expression of double antibiotic resistance.

Materials and Methods

The materials used and methods employed in the preparation of nuclear matrix from fetal calf thymic nuclei are nearly identical to those described for rat thymus (6) except that the starting material consisted of 20 gm tissue. Fetal calf thymus was obtained from Pel freeze company and stored frozen until use. The assay procedure for recombination, using pJH 200 as a substrate, has also been described (6). The restriction enzymes Pvu II, HgiAl and Sal I were the products of Boehringer Corporation and were used in accordance with the protocols described by the manufacturers. All the restriction fragment analyses were carried out on 1% agarose gels.

Results and Discussion

Nuclear matrix from fetal calf thymus: Since the quantity of nuclear matrix that one can obtain from the rat thymus source is quite limited, we searched for and found fetal calf thymus nuclei as an alternate source for it. The nuclear matrix prepared from calf nuclei, following the protocol used for rat nuclei (6), contained terminal deoxynucleotidyl transferase, an exonuclease, DNA ligase, DNA polymerase and several DNA binding proteins (data not shown). The Calf nuclear matrix preparation exhibited many properties that seemed quite similar to those reported for rat thymus matrix (7). Most importantly, calf

TABLE 1. V(D)J recombination activity in various thymic nuclear fractions

Nuclear fraction	Amp^r	Amp^r + Cam^r	% recombination
Control	2344	0	0
DNA depleted nuclei	1150	18	1.5
Total Nuclear Extract (2 M NaCl)	850	60	0.5
Nuclear matrix	1251	376	30.0
Soluble Nuclear matrix extract (2M salt + sonication)	650	19	3.0

The subnuclear fractions of fetal calf thymus nuclei were prepared and the in vitro V(D)J recombination assay was carried out with these fractions using pJH200 as the recombination substrate as described (6). A control containing all the reagents except the fractions was also processed similarly. Percent recombination was determined according to: $(Amp^r+Cam^r/Amp^r)X$ 100. The number of transformants (Amp^r or $Amp^r+ Cam^r$) has been corrected for a plating dilution factor.

thymic nuclear matrix appeared to support the recombination of pJH 200 plasmid in vitro as judged by the appearance of doubly resistant colonies (Table 1). Furthermore, the presence or absence of deoxynucleotides did not significantly alter the recombination frequency (data not shown). Thus, both calf and rat thymic nuclear matrices appeared to contain the putative V(D)J recombinase system. One of the troubling observations in the recombination assay was the fact that growth of doubly resistant (Amp+Cam) clones required somewhat longer periods of incubation (around 30 h) in contrast to clones which grew in the presence of ampicillin alone. We, therefore, randomly picked twenty colonies from both Amp and Amp+Cam plates, isolated the plasmid DNA from individual clones and subjected them to the restriction enzyme analysis.

Restriction enzyme analysis of plasmids isolated from doubly resistant clones:

In the recombined plasmid, a 200 base pair long sequence representing transcription termination must be removed in order to permit the expression of the chloramphenicol acetyl transferase (CAT) gene which was present immediately downstream from the terminator in the parent plasmid. The rearranged gene sequences

in the plasmid are also reported(5)to generate a new site for HgiA1 restriction enzyme. Thus, a Hgi A1 digest of plasmid DNA would contain two new fragments (~ 700 and 900 base pairs in length). As an additional confirmation of recombination, a deletion of the 200 base long terminator sequence can also be monitored by examining the reduction in length of a specific restriction fragment generated by other enzymes such as Sal I and Pvu II. Sal I cleaves the parent plasmid and generates two fragements of 5 KB and 1.1 KB. The latter contains the transcription terminator. In the recombined plasmid, one would expect the length of this fragment to be reduced to below 1000 base pairs. In Pvu II digests, a 650 BP fragment seen in the control would be reduced to 450 BP in the recombined plasmid. The results of the analyses of the restriction fragments of the control and doubly resistant plasmid are shown in fig 1. (Results for one control and 3 representatives of recombined plasmids are shown). It is clear

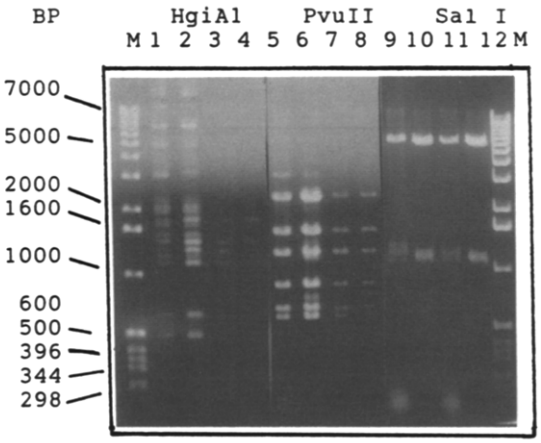

Figure 1. Restriction fragment analyses of pJH 200 DNA isolated from AMP^r and CAM^r clones:

Two hundred nanograms of pJH 200 DNA was incubated with fetal thymus nuclear matrix fraction in a standard reaction mixture (6). After 30 min at 37°C, the reaction was terminated by the addition of 10 mM EDTA and DNA was extracted by phenol:chloroform and precipitated with ethanol. An aliquot of this DNA was then used to transform E. coli (HB 101) and cells were plated on plates containing ampicillin and ampicillin plus chloramphenicol. After 30 h at 37°C, single colonies from plates were grown in 10 mL LB broth containing appropriate antibiotics and plasmid DNA was isolated. Plasmids were then treated with the desired restriction endonuclease and the products were resolved on 1% agarose gels. Lanes 1-4 show HgiA1 digestion products with 1 serving as a control plasmid and samples 2, 3 and 4 represent 3 different DNAs isolated from doubly resistant clones. Lanes 5-8 and 9-12 represent patterns of pvu II and sal I restriction enzyme digests using the above DNA samples with lanes 5 and 9 as controls. Lane M is a molecular weight marker lane. The numbers in the left hand margin indicate the migration patterns of known markers.

from the results that there is no difference in the restriction fragment lengths in the two sets. These results clearly suggest that the observed expression of the Cat gene did not result from a V(D)J specific recombination event but is probably mediated by some other undefined system. Results, very similar to ours, have also been obtained by Dr. M. Gellert using mouse thymus nuclear matrix as a test material (personal communication). In summary, the previously drawn conclusion concerning the successful *in vitro* recombination of DNA sequences, under the control of V(D)J signal sequence elements, is erroneous and caution should be exercised in the interpretation of the bacterial transformation assay when used to assess V(D)J recombination.

Acknowledgments: We thank Dr. Martin Gellert for alerting us to the potential problem in the assay system used and for the communication of results obtained with mouse thymus matrix.

References

1. Tonegawa, S. (1983) Nature 302, 575-581.

2. Blackwell, T. K. and Alt, F. W. (1989) J. Biol. Chem. 264: 10327-20330.

3. Alt, F. W., Blackwell, T.K., Depinho, R. A., Reth, M. G. and Yancopoulos, G. D., (1986) Immunol. Rev. 89: 5-30.

4. Hesse, J. E., Leiber, M. R., Gellert, M. & Mizuuchi, K. (1987) Cell 49: 775-783.

5. Leiber, M. R., Hesse, J. E. Mizuuchi, K. & Gellert, M. (1988). Curr. Top. Microbio. Immunol. 137, 94-99.

6. V. P. Dave, M. J. Modak, V. N. Pandey (1991) Biochemistry 30, 4763-4767.

7. Pandey, V. N., Dave, V. P. and Patil, M. S. (1989) Mol Biol. Rep 13, 179-184.