The kinetics of V–J joining throughout 3.5 megabases of the mouse Igκ locus fit a constrained diffusion model of nuclear organization

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Received 11 November 2002; revised 7 January 2003; accepted 8 January 2003

First published online 21 January 2003

Edited by Horst Feldmann

Abstract To gain insight into the nuclear organization of the mouse Igκ locus and how it may relate to the formation of synapses during recombination, we have studied the kinetics of rearrangement of different Vκ gene families to Jκ gene segments in the pre-B cell line, 103bcl2. Remarkably, Vκ gene families separated by more than 3.5 Mb from Jκ gene segments rearranged with nearly identical kinetics to those as close as 18 kb to Jκ gene segments. These results fit a model of nuclear organization in which the entire VκJκ region resides within a single nuclear subcompartment and is capable of exhibiting multiple reversible contacts through diffusion and Brownian motion.

Key words: Igκ; Recombination; RAG; Pre-B cell; Nuclear organization

1. Introduction

The mouse Igκ gene locus is the largest mutigene family yet identified with respect to genomic length, spanning more than 3500 kb (3.5 Mb) [1–6]. The locus is composed of 96 potentially functional Vκ gene segments, which reside upstream of four functional Jκ gene segments and the Cκ exon [5]. These Vκ gene segments belong to some 18 different families sharing ≥80% sequence identity. Members of a given Vκ gene family are semi-clustered but partially interspersed with members of other Vκ gene families [1,5].

During the differentiation of pre-B cells the Igκ locus becomes activated to undergo Vκ–Jκ joining, the gene rearrangement process necessary to produce Igκ light chains [7,8]. Depending on the orientation of the Vκ gene segment, rearrangement occurs either by deleting or inverting the DNA sequences that reside between the corresponding Vκ and Jκ segments participating in the recombination event [9]. Vκ–Jκ joining is mediated in part by the recombination-activating gene (RAG1/RAG2) products, which are required to create double-stranded breaks at the recombination signal sequences (RSSs) adjacent to each Vκ and Jκ region [10]. The RSSs of Vκ and Jκ regions possess 12 and 23 bp spacers, respectively, between their conserved heptamer and nonamer recognition sequences [11]. The formation of a productive synapse occurs preferentially between RSSs having dissimilar spacer lengths and is thought to proceed by RAG1/2 proteins first assembling on RSSs with 12 bp spacers (e.g. Vκ gene segments), followed by a search for its 23 bp spacer-containing partners (e.g. Jκ gene segments) [12]. Furthermore, formation of synaptic complexes between RSSs by the recombination machinery occurs prior to the cleavage and ligation reactions [13–15]. Given the immense size of the mouse Igκ locus, it is interesting to consider the mechanism by which a synapase might form, and how nuclear organization and higher-order chromosome structure might affect this process.

The genome is organized non-randomly in the nuclei of living cells (for reviews, see [16,17]). Individual chromosomes occupy specific territories [18], and different chromatin segments exhibit movement limited to their own nuclear subcompartments [19,20]. Within a given subcompartment DNA segments can diffuse by Brownian motion [19]. This type of nuclear DNA sequence mobility has been termed 'constrained diffusion' [19]. DNA mobility may also be confined to separate chromatin domains by the specific attachment of chromatin segments to nuclear substructures, such as the nucleoli, the nuclear periphery, and the nuclear matrix [21,22]. With reference to recombination, it is noteworthy that DNA segments most frequently involved in chromosome translocations are localized very close to each other in the interphase nucleus [16]. Furthermore, recent results from fluorescence in situ hybridization experiments reveal that the IgH gene locus exhibits a large-scale compaction in apparent preparation for V-to-DJ rearrangement [23].

In order to gain possible insight into the nuclear organization of the Igκ gene locus and the mechanism of synapse formation, we have utilized the pre-B cell line 103bcl2 [24] to explore the kinetics of Vκ–Jκ joining throughout the locus. This cell line is well suited for these studies because it is transformed by a temperature-sensitive mutant of the Abelson murine leukemia virus, permitting the induction of relatively high levels of both germline and RAG1/2 transcription, followed by recombination of the Igκ genes, upon incubation at the non-permissive temperature [24]. In addition, the endogenous Igκ alleles are largely in the germline configuration prior to the temperature shift. To investigate Vκ–Jκ joining, we have employed a modification of the polymerase chain reaction (PCR) assay of Schlissel and Baltimore [25] utilizing family-specific Vκ gene primers [1]. Our analysis takes advantage of the fact that coding joints rapidly form after DNA cleavage at the RSSs [26,27], and hence the rate of Vκ-Jκ joining should be a direct reflection of the rate of synapse formation. Our

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Abbreviations: RAG, recombination-activating gene; RSS, recombination signal sequence

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doi:10.1016/S0014-5793(03)00040-1

results reveal that Vκ regions as close as 18 kb or as far as 3500 kb from Jκ1 rearrange with nearly identical kinetics upon induction of RAG1/2 proteins. We discuss these results in the context of various models of nuclear organization including the 'constrained diffusion' model.

2. Materials and methods

2.1. Cell culture

The mouse pre-B cell line, 103bcl2, was obtained from Dr. Naomi Rosenberg (Tufts University School of Medicine) [24]. Cells were cultured at 34°C in RPMI medium 1640 supplemented with 10% fetal bovine serum (HyClone Laboratories), 2.0 mM glutamine, 50 μM β-mercaptoethanol, 50 μg/ml gentamicin and 700 μg/ml G418. To induce V–J joining, cells were heat-shifted to 39.5°C for the lengths of induction as indicated in the text. Where indicated, cells were synchronized by exposure to nocodazole (50 μg/ml) for 24 h, followed by 8 h of recovery in fresh medium prior to the temperature shift. Synchronization was confirmed by fluorescence-activated cell sorting.

2.2. PCR amplification assays for Vκ–Jκ joining

The family-specific forward Vκ primers used were:
Vκ1, 5′-TTGCCTGTTAGGCTGTTGGTGCTG-3′;
Vκ2, 5′-GCCCAGTTCCTGTTTCTGTTAGTGC-3′;
Vκ4/5, 5′-CARGTGCAGATTTTCACTTCCTGC-3′;
VκOx, 5′-GATTTTCAGCTTCCTGCTAATCAGTGC-3′;
Vκ8, 5′-TGTTCTGGGTATCTGGTACCTGTG-3′;
Vκ9/10, 5′-CAGATTTTTGGCTTCTTGTTGCTCTTG-3′;
Vκ19/28, 5′-ATGAAGTCACAGACCCAGGTCTTC-3′;
Vκ20, 5′-GCCTTCTTCTCCTCTGTGTCTCTG-3′;
Vκ21, 5′-TGCTGCTGCTCTGGGTTCCAGGTG-3′;
Vκ22, 5′-CTATTTCTTATTGTAGGTGCCTCGTG-3′;
Vκ23, 5′-GCCAGCCAGAGTATTAGCGACTACT-3′;
Vκ33/34, 5′-TCCTTTTCAACTTCTGCTCTTCCTGC-3′.

Primary references for Vκ gene sequences are cited in [28], with the exception of Vκ20 [29] and Vκ33/34 [30]. For assay of rearrangement to Jκ1 and Jκ2 only, the reverse primer used was 5′-ATGTACACCACAAACTCATACAAAGG-3′. For analysis of rearrangement, genomic DNA samples were purified as described elsewhere [31]. Reactions in 50 μl were performed with 100 ng DNA template, 200 μM of each deoxynucleotide and 5 pmol of each primer at 3 mM Mg^{2+} in 10 mM Tris, 50 mM KCl, pH 8.3 using 5 U of Taq polymerase for 1 min at 94°C, 2 min at 55°C, 1 min at 72°C for 30 cycles. Samples were separated on 1% agarose gels and blotted for Southern hybridization using ^{32}P-labeled 3′ primer as a probe [32]. For assay of rearrangements to all Jκ regions a published technique was used but without restriction enzyme digestion [31].

Table 1
Features of the Vκ gene families studied in the present investigation[a]

Vκ gene family	Relative location in the locus	Number of members/ orientations[b]
2	5′	3+0−
20	5′	2+0−
9/10	5′	6+2−
1	5′	3+2−
33/34	central	1+1−
4/5	central	1+26−
ox	central	1+0−
23	3′	0+5−
8	3′	0+8−
22	3′	0+1−
19/28	3′	2+7−
21	3′	9+0−

[a]Data compiled from [5].
[b]The (+) orientation refers to V regions where, when they become rearranged, the intervening DNA sequence will be deleted. By contrast, the (−) orientation refers to V regions where, when they become rearranged, the intervening sequence will become inverted.

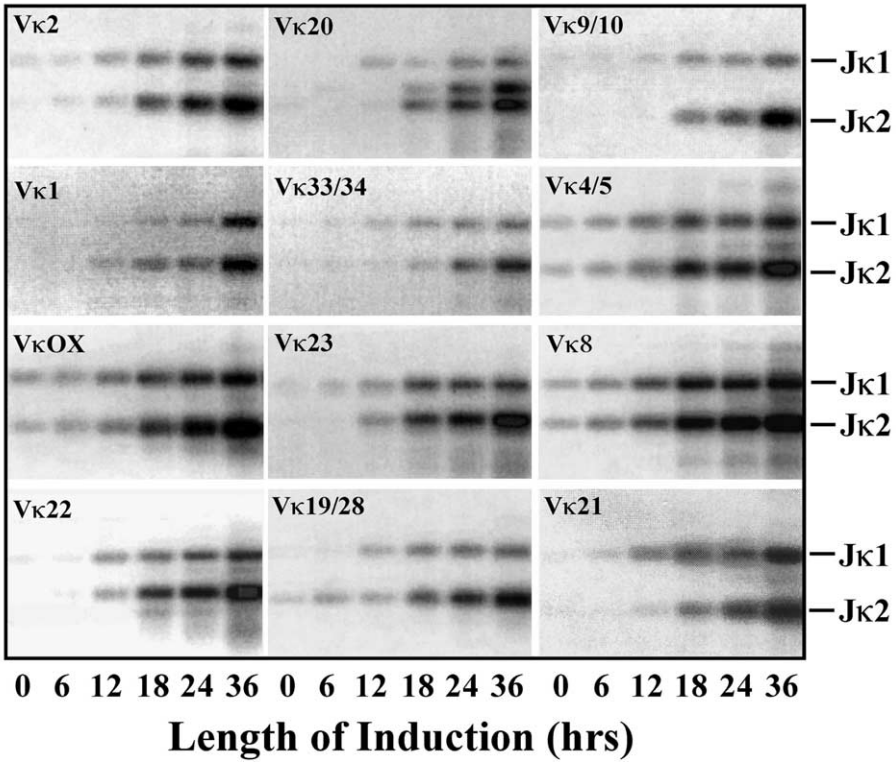

Fig. 1. Robust and rapid rearrangement of different Vκ gene families. Cells were shifted to the non-permissive temperature and samples were taken at the indicated times for PCR assays of rearrangement of the depicted Vκ gene families. Samples were separated by gel electrophoresis and transferred to membranes for hybridization with ^{32}P-labeled primer probes.

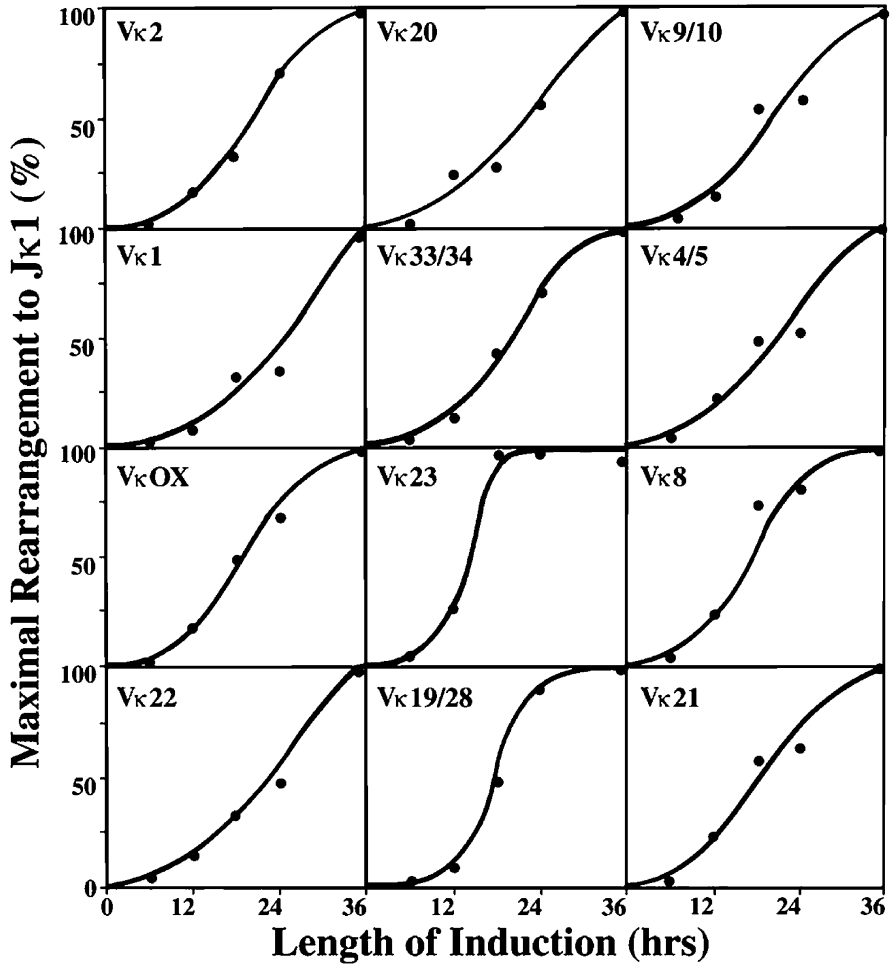

Fig. 2. Kinetics of the rearrangement of different Vκ gene families to Jκ1. The data for rearrangement to Jκ1 from Fig. 1 were quantitated with ImageQuant software. Plotted are the relative levels of rearrangement after subtraction of zero time background from all the corresponding samples and normalization to 100 for the maximal level of rearrangement seen among the corresponding samples.

3. Results and discussion

3.1. Overall experimental strategy

As shown in Table 1, we selected Vκ gene families for rearrangement assays that are represented by single members in the positive (2-phenyloxazolone, ox) or negative orientation (22), multiple members all in the positive (2, 20, 21) or negative orientation (8,23), and those which possess multiple members in both orientations (1, 9/10, 19/28, 33/34). These families are positioned in the distal 5′ region, the central region, and the 3′ Jκ proximal region in the locus and are representative of some 80 Vκ genes out of a total of 96 (Table 1). We have previously demonstrated that the Vκ gene primers chosen for these studies are highly family-specific because we have used them successfully in the past to clone and physically align the locus from a genomic library contained in yeast artificial chromosomes [1].

3.2. Vκ gene segments dispersed throughout the locus rearrange with nearly equal kinetics

Initially, we assayed for rearrangement of different Vκ gene families to Jκ1 and Jκ2. Regardless of the distance of the Vκ gene family from these Jκ regions, rearrangement detectable over the background seen in uninduced cells occurred within 12 h from the temperature shift and peaked for most families at 36 h (Fig. 1). To more readily compare these kinetics, we have quantitated these phosphoimages using ImageQuant software and plotted the data for rearrangement to Jκ1 after subtracting the background of rearrangement seen in uninduced cells. As shown in Fig. 2, the kinetics of rearrangement for all Vκ gene families studied were nearly identical, except for Vκ23 and Vκ19/28, which peaked earlier at 18 and 24 h, respectively. The kinetics for rearrangement to Jκ2 were also very similar for the different Vκ gene families, but often lagged behind slightly the rearrangement to Jκ1 (data not shown). Previous studies have shown that increases in RAG1/2 mRNAs occur from 4 to 6 h after the temperature shift [24] and clearly a period of additional time must be required for the protein products to accumulate upon translation of these mRNAs. The nearly identical kinetics for rearrangement seen here suggest that every Vκ gene family studied is accessible to assemble newly synthesized RAG1/2 proteins on their RSSs [12], and after this assembly the complexes have nearly equal chances with time to find Jκ region RSSs for synapase formation, followed by rapid subsequent cleavage and ligation reactions [13–15,26,27]. It remains to be

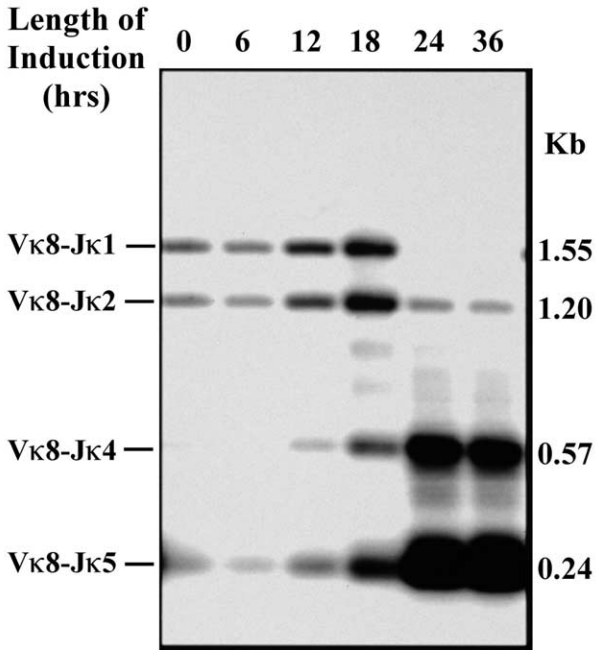

Fig. 3. Evidence for repeated recombination of Vκ8 family members. Cells synchronized by reversible nocodazole treatment were shifted to the non-permissive temperature and samples were taken at the indicated times for PCR assays of rearrangement. The final extension step used a ^{32}P-end-labeled primer.

determined whether such equal accessibilities, which pertain to the mouse pre-B cell line, 103bcl2, could possibly differ in primary cells or other cell lines.

3.3. Usage of Jκ regions follows the preference Jκ1 ≥ Jκ2 ≫ Jκ4 ≥ Jκ5 and recombination goes to apparent completion

We also studied rearrangements to the other functional Jκ gene segments, Jκ4 and Jκ5. It is known that incubation of 103bcl2 cells at the non-permissive temperature results in G1 cell cycle arrest [24], the same cell cycle period in which V–J joining occurs due to the induction and post-translational stabilization of RAG1/2 proteins [33]. Therefore, we used synchronized G1 phase cells to better study the kinetics of Jκ usage. Fig. 3 shows the results of such an experiment for the Vκ8 gene family, which first rearranges to Jκ1 and Jκ2, but with prolonged induction rearrangements to Jκ4 and Jκ5 dominate. Comparison of the kinetics of Vκ8 rearrangement between random exponential cultures and synchronized cells reveals that to Jκ1 usage peaks about 18 h earlier in the synchronized cells (compare data of Figs. 2 and 3). However, the earliest rearrangements detectable over zero time background were still only after 12 h (Fig. 3). Similar assays for Vκ2, 20, 4/5, 23, 19/28 and 21 with synchronized cells also revealed very similar kinetics and extensive usage of Jκ4/Jκ5 after prolonged induction (data not shown). Thus, rearrangement goes to apparent completion within the locus, consistent with a lack of allelic exclusion along with the repeated rearrangements noted by others in Abelson-virus transformed pre-B cell lines [34,35]. Single-cell PCR assays reveal that B lymphocytes from mice also prefer Jκ1,2 during early rearrangement events [36], which allows for later receptor editing to Jκ4,5 [37,38].

3.4. How does the mechanism of synapase formation relate to models of Igκ gene locus nuclear organization

In conclusion, we have found that the rearrangement process throughout the 3.5 Mb mouse Igκ locus is rapid and robust with all Vκ and Jκ gene segments studied being accessible to the recombination machinery. If a processive searching for RSSs occurs along the chromatin fiber, starting first with Vκ gene RSSs [12], followed by linear scanning/tracking along a chromatin fiber to find a Jκ RSS, then one would expect the rearrangement kinetics to differ as a function of the distance of the Vκ gene segment from the Jκ region. This result was not observed. Another possible model is a non-random looping model, in which certain Vκ regions might be organized closer than others to the Jκ region, through higher-order folding or looping of chromatin fibers. This type of organization would again predict differences in the kinetics of rearrangement between Vκ families, which would not necessarily be related to their linear distances from the Jκ region. Again, such a result was not obtained, with the exception of the favored Jκ region usage. The preference for closer Jκ regions could simply reflect differential accessibilities of their corresponding RSSs, and/or be a result of the downstream nuclear matrix association region [22], which would be predicted to progressively reduce the mobility of DNA sequences the closer they are to the attachment site [21]. In any case, our results are consistent with considerable mobility of chromatin throughout the Igκ gene locus, or preformed heterogeneity in the loop domain organization among different pre-B cells (or both). Because the entire locus can undergo rearrangement after long periods of RAG1/2 protein induction, we favor a 'constrained diffusion' model of nuclear organization [19], in which the entire VκJκ region resides within a single nuclear subcompartment and is capable of forming synapses through multiple reversible contacts by diffusion and Brownian motion. This view of a single nuclear subcompartment for the Igκ gene locus is also consistent with the observation that distal segments of the IgH gene locus become spatially co-localized in nuclei of pro-B cells [23].

Acknowledgements: We thank Katherine Brekke for a critical review of this manuscript and Piotr Widlak for computer graphics. This research was supported by Grants GM29935 from the National Institutes of Health and Grant I-823 from the Robert A. Welch Foundation to W.T.G.

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