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Immunology

Immunoglobulin heavy chain enhancer is located near or in an initiation zone of chromosomal DNA replication

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ABSTRACT **In several animal viruses, enhancers have been implicated in both DNA replication and transcriptional activation. The linkage of the two mechanisms appears intimate, in that common DNA binding factors can be shared. The immunoglobulin heavy chain (*Igh*) intronic [heavy chain joining region (J_H)–μ chain constant region (Cμ)] enhancer (Eμ) is required for tissue-specific transcription of *Igh* genes and is essential for somatic recombination of diversity (D) and J segments. We show here that Eμ is located at or near an origin of chromosomal DNA replication, which is more active in B lymphocytes than fibroblasts. Eμ does not fulfill two criteria demonstrated for some cellular origins. Eμ can initiate but not maintain autonomous replicating activity in B cells. Eμ is unable to impart early replication timing to a transfected VDJ–Cμ *Igh* locus in B cells. Instead we propose that Eμ-associated *ori* activity contributes to tissue-specific *Igh* expression through local effects on chromatin structure leading to subsequent accessibility of transcription and/or recombination factors for the enhancer.**

Gene expression in eukaryotic cells is controlled by trans-acting factors and cis-acting transcriptional elements. Some transcription factors have been shown to activate DNA replication when the factor-binding sequences are located in the vicinity of a minimal origin (1, 2). Activation of DNA replication by transcription factors is likely to be evoked through a change in chromatin structure (1, 3). The initiation events of transcription and DNA replication may be closely related in that they both necessitate the transient breakdown of higher-ordered chromatin.

All detailed analyses of the relationship between transcription and DNA replication have been performed in viral systems (for review, see ref. 4). The immunoglobulin heavy chain (*Igh*) locus provides an excellent system to address this issue for a cellular gene because its mechanism of expression has been studied extensively (for review, see ref. 5). Transcription of *Igh* genes is regulated by elements within the heavy chain variable region (V_H) promoter and the *Igh* enhancer (Eμ). Some cis-acting sites, such as the octamer, are shared between Eμ and promoter and play a key role in lymphoid-restricted expression (6, 7). Oct-1, a ubiquitous octamer-binding factor, can activate adenovirus DNA replication (8), suggesting that Eμ-associated motifs may be involved in more than transcriptional enhancement.

S phase of the cell cycle can be divided into stages, with actively expressed genes often replicated in early S phase and repressed genes replicated during late S phase (for review, see ref. 9). Studies of DNA replication timing of the *Igh* locus revealed that, in nonlymphoid cells, DNA replication initiates far downstream [3′ to the α chain constant region (Cα)] and V_H segments replicate late in S phase (10). In lymphoid cells, however, productively rearranged V_H genes and more Eμ-proximal C regions replicate as early as or earlier than Cα replicates in nonlymphoid cells (11). These data suggest that the *Igh* locus has another origin(s) that could be developmentally regulated.

Eμ is active throughout B-cell differentiation, even prior to complete (V to DJ, where D is diversity and J is joining) assembly of immunoglobulin V_H genes (12, 13). Consistent with its J_H-proximal location, Eμ is essential for activating the locus for recombination. Transgenic studies (14) have demonstrated that Eμ can act dominantly in cis to initiate the D-to-J rearrangement in lymphoid tissues. Transfection of a recombination test gene containing Eμ and the metallothionein promoter into plasmacytoma cells showed that the promoter, but not the enhancer, was dispensable for efficient V–J joining (15). These studies suggest that what is required for rearrangement is not Eμ-mediated transcription *per se* but an open chromatin state.

The above considerations prompted us to search for an origin of chromosomal DNA replication in the Eμ region of the *Igh* locus. Our results revealed that Eμ is at or within the vicinity of an initiation zone of chromosomal DNA replication that is more active in B cells than in fibroblasts. We propose a model for a functional relationship between Eμ-associated DNA replication and Eμ-dependent transcriptional and recombinational activities.

MATERIALS AND METHODS

Semiquantitative PCR. Primer pairs and hybridization probes are 24 bases long and composed of 13 G-C pairs. Positions of each primer pair and probe are as follows: J-specific, 5′ primer (nt 913–936), 3′ primer (nt 1168–1191), and probe (nt 1017–1040); E-specific, 5′ primer (nt 2553–2576), 3′ primer (nt 2822–2845), and probe (nt 2710–2733); switch (S)-specific, 5′ primer (nt 5501–5524), 3′ primer (nt 5779–5802), and probe (nt 5542–5565). These positions are according to our numbering system (GenBank accession no. X57331). Thirty cycles of PCR were performed (16). PCR products were alkaline-denatured, slot-blotted, and hybridized with each oligonucleotide probe at 10°C below the theoretical melting temperature. The radioactivity from each hybridized slot was quantified by ion emission (Betagen, Waltham, MA).

Purification of Nascent DNA. Purification of nascent-strand DNA was performed essentially as described (17). Approximately 1×10^8 cells were labeled with [^{3}H]deoxycytidine (2 μCi/ml; 1 Ci = 37 GBq) and 5-bromodeoxyuridine (BrdUrd; 20 μM) for 10 min. The resulting high molecular weight DNA was size-fractionated on 5–20% (wt/vol) linear alkaline sucrose gradients. Radioactivity in a 4% volume of each fraction was measured by liquid scintilation. BrdUrd and ^{3}H-

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Abbreviations: Igh, immunoglobulin heavy chain; J, joining; C, constant; Eμ, *Igh* enhancer; D, diversity; V, variable; H, heavy; L, light; Cμ, V_H, etc., μ chain C region, H chain V region, etc.; Fr, fraction(s); S, switch; ARS, autonomously replicating sequence.

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labeled DNA was isolated by two rounds of immunoprecipitation with anti-BrdUrd monoclonal antibodies. The specific activity of the purified DNA fraction was increased by 400- to 800-fold over the initial specific activity. Size distribution of nascent DNA was determined by alkaline agarose gel electrophoresis and Southern blot analysis with ^{32}P-labeled chromosomal DNA. The concentration of purified nascent DNA was measured by fluorometry (18).

Transient Transfections and *Dpn* I Assays. Transfection of lymphoid cells and nonlymphoid cells was carried out by electroporation (19) and by calcium phosphate methods (20), respectively. At 48 h after transfection, low molecular weight DNAs were extracted (21) and linearized by digestion with *Bam*HI (22). Fragments were separated on an 0.8% agarose gel and hybridized with control DNA probe whose sequence is identical in both test and control plasmids.

Semiconservative Replication Assay. At 2 days after transfection, cells were washed with fresh medium and labeled with BrdUrd (12.5 μg/ml) for 24 h. Plasmid DNA was purified and BrdUrd-substituted and unsubstituted DNAs were resolved on native CsCl gradients (23). The DNAs were electrophoresed and blot-hybridized to vector DNA. The banding densities of light–light (LL) and heavy–light (HL) DNAs are determined by electrophoresing marker DNAs in parallel.

Replication Timing Assay. M12.4 B lymphoma cells were stably transfected with a 15-kbp VDJ–Cμ construct as described (24). Cells were labeled with BrdUrd as described above, then stained with chromomycin (CA3, 20 μg/ml), and sorted on a FACStar III (Becton Dickinson) adjusted to detect nuclei excited at 457 nm at a flow of 2000 cells per sec (25). Based on DNA content, $\approx$0.5–2 $\times$ 10^6 cells were collected in six windows. Isolation, gradient separation, and slot hybridization of replicated and unreplicated DNA from the cell-cycle fractions was performed as above. A 1.2-kb *Bam*HI fragment from plasmid pLLn2R (24) was used as a VDJ-specific probe (S107) and a 3.2-kb *Sac* I fragment was used as α-globin genomic probe.

RESULTS

Strategy for Mapping an Origin of Chromosomal DNA Replication. Several general approaches have been developed to map chromosomal origins (16, 17, 26–31). The PCR method (16, 17) was chosen in this study because the entire DNA sequence from VDJ_1 to Cμ is known. In principle, this method determines the distance of specific DNA sequence markers to an origin by fractionating nascent DNA chains according to size and identifying the shortest nascent DNA chains (i.e., most origin-proximal sequences) by PCR amplification and hybridization with ^{32}P-labeled probes directed against the specific sequence markers. We chose the B-cell leukemia line BCL_1 for our analysis because it is representative in many respects (32) of a murine B lymphocyte and offers the operational advantage of containing a single productively rearranged VDJ–Cμ allele locus; the other apparently was deleted on adaption to culture (33).

Purification of Nascent DNA. Nascent DNA from asynchronously growing cells was labeled with [^{3}H]deoxycytidine and BrdUrd, size-fractionated on an alkaline sucrose gradient, and purified by anti-BrdUrd antibodies (17) (Fig. 1). To avoid contamination of Okazaki fragments and unreplicated high molecular weight DNA, fractions (Fr) 1, 2, 12, and 13 were discarded, and Fr 3–11 were relabeled Fr 1–9 in Fig. 1*B*. Fr 9 does not contain nascent DNA fragments >100 kb. Incorporation of [^{3}H]deoxycytidine into nascent DNA depends on the size of the DNA in a fraction (Fig. 1*A*), except for Fr 1, in which Okazaki fragments were presumably present. The intensities of hybridized bands increased proportionally with DNA length (Fig. 1*B*). This suggested that

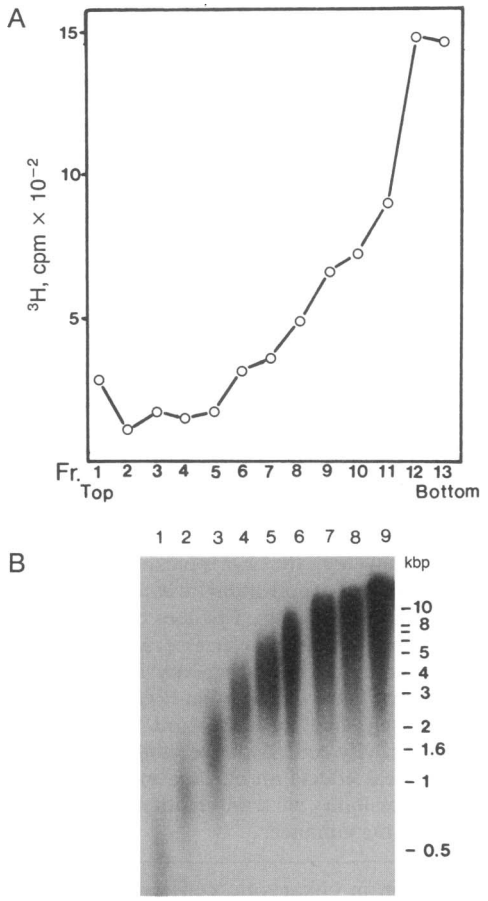

FIG. 1. Size separation and size determination of pulse-labeled DNA. (*A*) BrdUrd- and ^{3}H-labeled DNA was size-fractionated in an alkaline sucrose gradient. The gradients were collected in 13 fractions from the top to the bottom, measured for ^{3}H incorporation, and plotted. (*B*) Fr 1, 2, 12, and 13 were discarded and the remaining fractions were renamed to Fr 1–9. The renamed fractions were purified by immunoprecipitation with anti-BrdUrd, separated on alkaline 1.0% agarose gel, and hybridized with ^{32}P-labeled BCL_1 chromosomal DNA. Marker DNA is the 1-kb DNA ladder (BRL). The average DNA size was determined by comparison of the migration of the hybridized bands with marker DNA.

the number of nascent DNA molecules were approximately the same in each fraction. Since these results were very similar to those reported (17), we concluded that the conditions shown were optimal to quantitate the relative amount of specific sequence in a given sample and to accurately size-fractionate nascent DNA.

Eμ Is Located Near or in the Initiation Zone of Chromosomal DNA Replication. To map a chromosomal origin, we established semiquantitative PCR conditions within the range of at least 0.5–32 ng (data not shown). Each primer set had a similar efficiency and was designed to avoid binding simultaneously to Okazaki fragments. The purified nascent DNA within each fraction was separately amplified at the same time with primer-pair J, located between J_H2 and J_H3; primer-pair E, just 5′ to Eμ; and primer-pair S, 3′ to the μ chain switch region (Sμ). Hybridization signals of E-specific sequences were high in every size fraction of anti-BrdUrd-enriched nascent DNA, whereas signals of J- and S-specific sequences were significantly decreased in the shortest fractions (Fig. 2*A*). This indicated that the copy number of E-specific sequences was high in the smaller-size fractions relative to those of S- and J-specific sequences. In addition, the presence of E-specific sequences in the shortest nascent fragments indicated that Eμ is in the initiation zone of

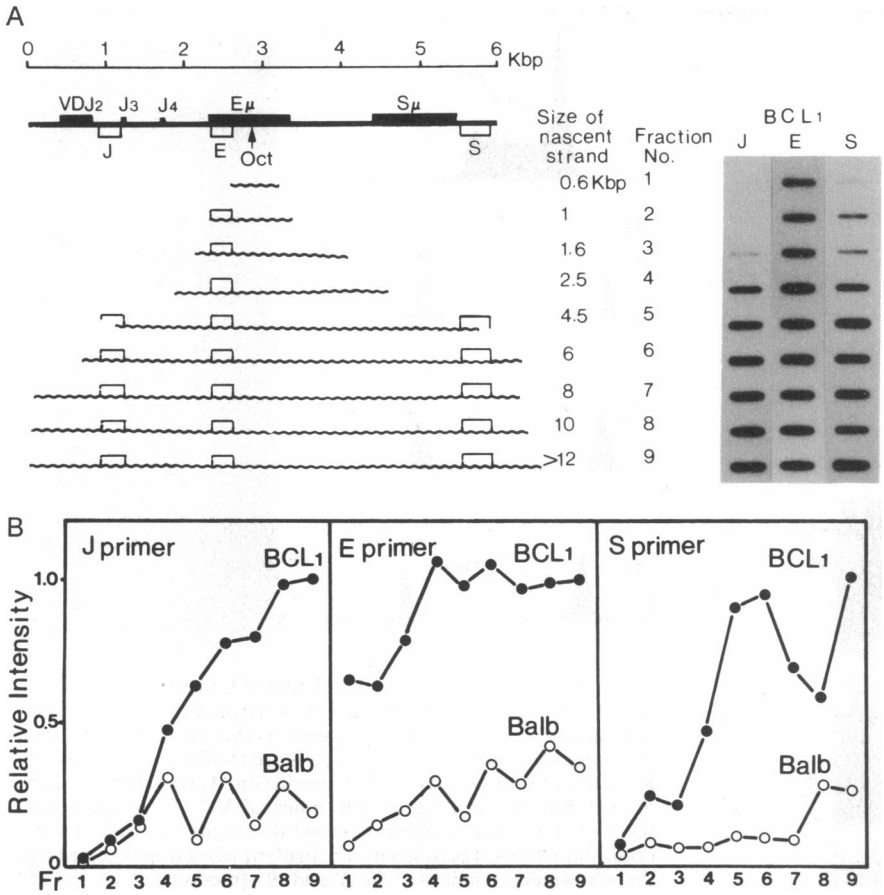

FIG. 2. Eμ is located in or near the initiation zone of a DNA replication origin. (*A*) Mapping a DNA replication origin within the *Igh* locus. (*Left*) The relevant part of the *Igh* gene locus with the octamer element (Oct) within Eμ noted. Open boxes indicate J, E, and S PCR amplifications of 278, 292, and 301 bp, respectively. The alkaline-sucrose-gradient-fractionated anti-BrdUrd-enriched nascent strands are represented under the map. The open boxes are hypothetical PCR-amplified regions whose lengths are consistent with the slot-blot hybridization data (*Right*). Purified nascent DNA in each fraction (e.g., 2 ng in Fr 1 to 25 ng in Fr 9) was separately amplified with each primer set and slot-blot-hybridized with the relevant probe. The radioactivities of each slot were measured by β-scope. The average DNA size of each fraction was obtained from the experiment in Fig. 1. (*B*) The Eμ origin shows cell-type preference. Relative amounts of specific amplification/hybridization in BCL_1 vs. BALB 3T3 are compared. All procedures in BALB 3T3 are the same as in BCL_1 cells. The radioactivity obtained from the largest DNA (Fr 9) in BCL_1 is assigned as 1.0 and the activities of each size fraction are plotted as a function of size. Sizes of nascent strands increase with increasing fraction number.

chromosomal DNA replication. However, in the shortest fragments (Fr 1–3), S signals were higher than J signals, although J-specific sequences are located ≈1 kbp closer to the E-specific sequences (see *Discussion*). Additional amplification with primer pairs 5′ (VDJ) and 3′ (Cμ) to those in Fig. 2*A* gave the expected low intensities (data not shown). Thus the relative intensities of signals and the relative physical locations of J and S regions suggest that the center of the initiation zone may lie just downstream of the Eμ region.

The Eμ-Associated Origin Appears to be More Active in B Cells. Eμ enhances transcription in a tissue-specific manner. If DNA replication plays a role in this specificity, the origin activity we have mapped might show similar restriction. To test this possibility, the equivalent analysis was performed using the same set of primers in BALB 3T3 fibroblasts. We found that the quality of purified nascent-strand DNA for 3T3 cells was similar to that of BCL_1 cells, as indicated by similar specific activities of at least the largest size fraction (data not shown). Each sucrose fraction of BALB 3T3 DNA and the largest fraction of BCL_1 DNA were amplified simultaneously by each primer set. All gradient-sized anti-BrdUrd-enriched fractions of nascent BALB 3T3 DNA showed consistently lower levels of newly replicated DNA irrespective of their similar intensities to BCL_1 as seen in the amplification of unfractionated chromosomal DNA. E-specific signals in BALB 3T3 were also enriched in the shorter fractions but to a significantly lower extent than in BCL_1. The relative intensities, plotted in Fig. 2*B*, showed that the copy number of newly replicated E sequences is ≈10-fold more abundant in BCL_1 than in BALB 3T3. These results suggest that the Eμ-associated origin may be more active in B cells. Although J- and S-specific signals were consistently more variable in 3T3, J intensities were always higher than S intensities, which is the reverse of their relative intensities in BCL_1, but both were depleted in the shortest fractions (Fig. 2).

The Eμ-Associated Origin Cannot Mediate Conventional Autonomously Replicating Sequence (ARS) Activity. ARS activity has been demonstrated for several chromosomal origins. Thus B cells and nonlymphoid cells were cotransfected with Eμ and the parental pUC plasmid (as a control) and then DNA from these cells was subjected to a *Dpn* I resistance assay (23) (Fig. 3*A*). The results in B cells suggested that a ARS element is present on Eμ (Fig. 3*A*, lanes 5 and 6). The activity was not detectable in NIH 3T3, BALB 3T3, CV-1, or HeLa cells (lanes 1–4, respectively). Although this putative ARS activity was low, no *Dpn* I-resistant bands were obtained from subclones derived from regions on both sides of Eμ (lanes 7 and 8).

As an independent test, a semiconservative replication assay (23) was performed (Fig. 3*B*). Eμ- and control plasmid-transfected BCL_1 cells were labeled with BrdUrd. When incorporated into replicating DNA, BrdUrd increases the density of the replicated molecules (HL and HH) and causes them to band at a density higher than that of unsubstituted (LL) DNA. In BCL_1 cells, where 15% of the positive control polyomavirus plasmid DNA (pPμ) and 3% of the negative control (pUC) DNA were density-labeled, 10% of Eμ (plgHe) DNA migrated into the HL or the HH fraction.

A more rigorous criterion for assigning ARS activity requires not only that the DNA replicate but also that it be maintained in the nucleus as a plasmid. To investigate extrachromosomal retention of Eμ activity, pEH (Eμ in a hygromycin B-resistance gene-containing pBR vector)-transfected cells were maintained for 3 weeks under drug selection. A semiconservative replication assay with these long-term cultured cells failed to detect Eμ DNA in the extrachromosomal fraction; most was integrated into the chromosome (data not shown). From these three experiments, we conclude that Eμ cannot support conventional ARS activity. That is, Eμ can initiate weak autonomous

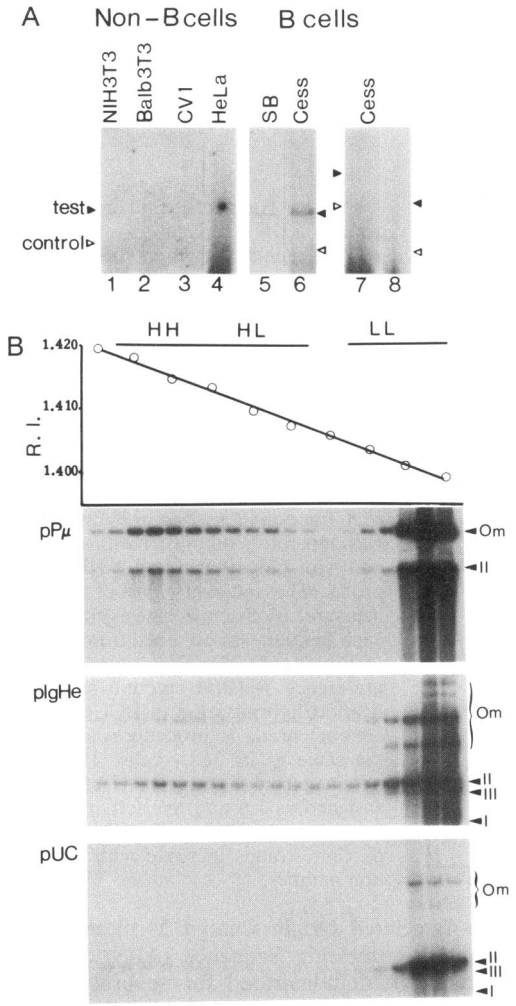

FIG. 3. Measurement of ARS activity of the Eμ in B cell lines. (*A*) *Dpn* I-resistance assay. Solid and open arrowheads indicate linearized newly replicated test and parental vectors, respectively. pIgHe (0.9-kb Eμ-containing pUC vector) and pUC DNAs were cotransfected into NIH 3T3 (lane 1), BALB 3T3 (lane 2), CV-1 (lane 3), and HeLa (lane 4) cells by the calcium phosphate transfection method. A human pre-B-cell line, SB (lane 5), and a human Epstein–Barr virus-transformed lymphoblastoid line, Cess (lane 6), were transfected by electroporation. Two cotransfections into Cess cells were performed: pB2 (pBR322 containing the 7.6-kb Sμ and Cμ fragments) with pBR322 (lane 7) and pV_1C(CAT) vector containing bases −550 to 0 of the V_H1 promoter region] with chloramphenicol acetyltransferase (CAT) vector (lane 8). Parental vector DNA was used as a probe for Southern blot analyses. (*B*) BrdUrd density labeling of Eμ-containing plasmid and banding on neutral CsCl. Plasmid DNA was purified from transfected cells and density-fractionated. The density and DNA concentration in each fraction were obtained by refractive index (R.I.) and Southern blot analysis, respectively. The positive control vector pPμ contains the early region of polyomavirus and a functional Cμ gene (34) and is capable of autonomous replication in murine cells. Om, oligomers; I, II, and III, forms I, II, and III of the plasmid.

replication in B cells but is insufficient for maintaining a plasmid in an extrachromosomal state.

Eμ and Associated VDJ–Cμ Sequences Cannot Control Replication Timing. Constant regions downstream of rearranged VDJ segments replicate earlier in S phase than their unrearranged counterparts (11, 35). It was feasible that the origin activity we measured for Eμ might be activated after DNA rearrangement. Therefore, we tested whether Eμ and/or associated sequences that form a μ transcription unit are sufficient to control replication timing in a B-cell line. A

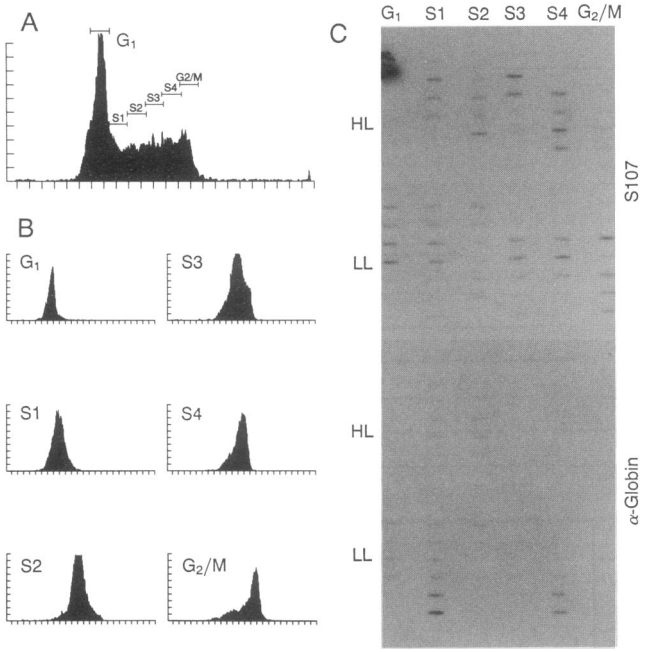

FIG. 4. Analysis of replication timing of transfected VDJ–Cμ in M12.4 cells. (*A*) Flow histogram of transfectants. Relative DNA content, measured by CA3 flourescence, is shown in a linear scale on the *x* axis and relative numbers of cells are shown on the *y* axis. (*B*) Resorting of cells, as indicated by brackets in *A*, revealed the purity of each fraction. (*C*) Replication times of VDJ–Cμ (S107 probe) (*Upper*) and mouse α-globin (*Lower*) determined by slot-blot hybridization. DNA was isolated from BrdUrd-labeled cells sorted into the six windows indicated in *A* and *B*, fractionated on a CsCl gradient, and blotted. Positions of HL and LL DNAs are indicated.

productively rearranged 15-kbp VDJ–Cμ construct (24) was stably transfected into a B-cell lymphoma, M12.4, that is devoid of an endogenous *Igh* locus. Transcriptionally active transformants (data not shown) were analyzed by the retroactive synchrony method (36).

Approximately 10^7 cells labeled with BrdUrd were sorted into six fractions by using equally spaced windows over the range of CA3 fluorescence from G_1 (2N) to G_2 + M (4N) (where N is the haploid number of chromosomes) (Fig. 4*A*). The purity of cells was checked by resorting (Fig. 4*B*). Equal quantities of fractionated DNA from each sort window were blotted and hybridized to a probe specific for the rearranged VDJ (S107, Fig. 4*C*). The S107 probe detected HL DNA across all S-phase fractions. Rehybridization of the blot to an α-globin probe, previously shown in B cells to replicate early (37), showed that the vast majority of α-globin replication is confined to the first half of S phase. Therefore, the sorting and gradient fractionation procedures were adequate to conclude that, unlike the endogenous rearranged locus, replication of the integrated transcriptionally active VDJ–Cμ "minilocus" was not confined to early S phase. This implies that Eμ along with the ≈7 kbp of colinear 5′ and 3′ sequences are insufficient to control replication timing.

DISCUSSION

We have demonstrated that Eμ is located near or in the initiation zone of chromosomal DNA replication, which may be more active in B cells. This is consistent with its requirement for transcriptional activation. A functional relationship between enhancers and DNA replication has been established in several viral systems, including polyoma (38). The polyoma enhancer has been reported to activate DNA replication to levels dependent on its distance from the origin;

the enhancer effect is eliminated if the distance is too great (39). We cannot formally conclude that Eμ is equivalent to a chromosomal origin. We can, however, minimally infer that Eμ is located very close to the initiation zone of a DNA replication origin. As in the above case, Eμ may activate origin activity.

A relationship between replication and transcription could account for the differing intensities of Eμ flanking regions (J and S in Fig. 2) in B cells and fibroblasts. Transcribed c-*myc* (40) and globin (41) genes are replicated in their transcriptional direction, whereas their quiescent germ-line counterparts are not. This implies that some origins are not bidirectional and their polarities are affected by transcription. The *Igh* locus is transcribed from J through S in B cells. Consistent with this, we observed higher replication signals in S than in J, even though J sequences are closer to the apparent origin region (E). There is no transcription of the locus in BALB 3T3. Accordingly, the more E-proximal J signal was higher than the more S-distal signal. Thus relative J and S intensities seem to be dependent on the presence of transcription, whereas the E signals were always highest irrespective of transcription. We suggest that Eμ is in the initiation zone of DNA replication in both cell types and that replication from it is not always driven bidirectionally.

There is a controversy as to whether an ARS element can serve as a chromosomal origin (42). Some chromosomally mapped origins function as ARS elements (29), although not all ARS elements serve as chromosomal replication origins (43). As with Eμ, some yeast ARS sequences contain matrix-associated regions (MARs) (for review, see ref. 44). We found that a 0.9-kb fragment containing Eμ and its associated MARs expressed a weak ARS activity in a cell-type-preferential manner. It was detectable by transient but not long-term replication assays. Perhaps Eμ can convey weak ARS activity in B cells but requires additional cis-acting sequences for maintenance of the plasmid in the replicating pool. However, immediate flanking sequences, at least, those contained within the ≈15 kbp spanning Eμ in a rearranged transcription unit, were insufficient to control replication timing in stable B-cell transfectants. In the extensively studied *CAD* and *ADA* system in CHO cells, timing control could only be localized only to within 30 kbp of a probe used to detect an ≈260-kbp replicon (45). Therefore, if ARS and/or timing control sequences are linked to Eμ, larger constructs will be required to detect them.

How might *ori* activity of Eμ manifest an effect on immunoglobulin gene expression? Several examples of stimulation of replication by transcription factors have been described (1, 46–48). A mechanism often proposed is change in chromatin structure, leading to an increased accessibility of the origin to replication proteins. That Eμ contains a VDJ recombination enhancing activity (14, 15) is consistent with its pattern of transcriptional enhancement in B and T cells. However, in most (15, 49, 50) but not all (51) experiments employing rearrangement substrates, the rate of transcription is not correlated with the frequency of recombination. CpG methylation was implicated in VDJ accessibility, but only if the test plasmids were replicating (52). A direct role for transcription seemed unlikely because there is little or no gene expression from fully CpG methylated plasmids (52, 53). We suggest a model in which Eμ-associated replication leads to a nuclease-sensitive VDJ chromatin structure (including targeted demethylation), which then allows recombination to occur.

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