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Elements in the Immunoglobulin Heavy-Chain Enhancer Directly Regulate Simian Virus 40 ori-Dependent DNA Replication

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In a previous study, we showed that the immunoglobulin heavy-chain (IgH) enhancer (IgHe) is near or in an initiation zone of chromosomal DNA replication, which is preferentially active in B cells (K. Ariizumi, Z. Wang, and P. W. Tucker, Proc. Natl. Acad. Sci. USA 90:3695–3699, 1993). This suggests the existence of a functional relationship between IgHe-mediated transcription and DNA replication. To test this theory, we utilized simian virus 40 (SV40) DNA replication as a model of chromosomal replication. IgHe or its operationally divisible domains (5′-En, core, and 3′-En) were introduced into SV40 minichromosomes (IgHe-SV40). Results of replication assays with IgHe-SV40 replicons indicated that the 5′-En and 3′-En activated or suppressed SV40 DNA replication regardless of the presence of SV40 enhancers or promoters in these replicons. The activity did not reside in IgHe core sequences. The results suggested that the 5′- and 3′-En regulated SV40 replication through direct interaction with the origin, not through suppression at the SV40 enhancer and/or promoter. In an effort to identify elements within the 5′-En motif that contributed to this effect, we found that the E site, but not μE5 and μE2 boxes, upregulated DNA replication. Our results provide another possible regulatory function for the 5′-En and 3′-En domains besides transcriptional suppression of IgHe.

Immunoglobulin heavy-chain (IgH) gene expression is controlled by *cis*-acting regulatory elements in the promoter (17, 26) and the enhancer (IgHe) (2, 15). A number of *trans*-acting factors, expressed either ubiquitously or tissue specifically, interact with motifs within the promoter (23, 35, 37, 38) and/or the IgHe (14, 28, 34) to regulate RNA synthesis. The promoter is indispensable for the initiation of tissue-specific transcription, but the role of the IgHe in this regard is controversial (reviewed in reference 37). After IgH gene activation, however, the IgHe is definitely required to maintain high levels of IgH transcription in B cells (18, 30). Thus, the IgHe is an essential *cis*-acting element in the regulation of IgH gene transcription. The molecular mechanism of enhancer function remains unclear. The ~0.9-kbp IgHe can be operationally divided into three domains (37) (Fig. 1C): a core segment, which contains several *cis*-acting activator motifs, and the flanking segments (5′-En and 3′-En). The 5′-En contains both activator (E and μE2) and negative-regulatory (μE5) motifs, whereas both flanking segments contain matrix association regions (MARs) and a putative repressor binding site (μNR).

Although the core origin of simian virus 40 (SV40) DNA replication contains all of the sequence elements that are absolutely required in *cis* for initiation of DNA synthesis, it is apparent that sequences outside the core can influence the efficiency of initiation (10, 21, 22). The presence of the SV40 enhancer and promoter adjacent to the core origin increases the efficiency of DNA replication in vitro at least 10-fold (22). Conversely, studies of the adenovirus and polyomavirus systems revealed that cell-type- and promoter-specific activation of transcription is dependent on DNA replication (11, 16). There is an increasing list of transcriptional activators shown to play important roles in DNA replication (6, 44). These findings strongly suggest a functional linkage between activation of transcription and DNA replication (27).

In the IgH locus, linkage of transcription and replication was inferred from timing studies demonstrating that germ line V_H genes replicate late, whereas productively rearranged, transcribed V_H genes replicate early in S phase (5). We (1) and others (20) have recently demonstrated that the IgHe is at or near an initiation zone of chromosomal DNA replication. The origin of DNA replication (IgHe-associated origin) is cell type preferentially regulated, as indicated by the observation of higher activity in B cells than in fibroblasts (1). IgHe-associated origin function may be involved in tissue-specific transcription and/or V(D)J rearrangement. Given the indication of a functional relationship between transcription and DNA replication in the IgH locus, three questions arise: (i) how is the IgH origin cell type preferentially regulated, (ii) how does IgHe contribute to the regulation, and (iii) is IgHe itself the IgH origin?

A reasonable strategy to address these issues would be to mutate IgHe sequences required to activate transcription and then determine whether the IgH origin activity is affected. At present, it is technically difficult to mutate only the IgHe region at the chromosomal level. Unlike some cellular origins, the IgH origin does not express conventional autonomously replicating sequence activity (1). This eliminates the possibility of carrying out in vitro mutagenesis of the IgH origin, as has been performed with the autonomously replicating sequence element of *Saccharomyces cerevisiae* (40) and with autonomously replicating viruses (22).

As an alternative approach to address the effects of IgHe

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sequences on origin regulation, we have examined their influences on SV40 DNA replication. SV40 has been exploited as a model for understanding the mechanism of DNA replication in eukaryotic cells (22). In this study, IgHe or its three operational domains were introduced into SV40 minichromosomes, and effects were measured by transient replication assays. We found that the 5′-En and the 3′-En (collectively referred to hereafter as the flanking region) can regulate SV40 origin of replication (ori)-dependent DNA replication. IgHe core sequences appear to have no effect. We also show that the E site (Fig. 1C), when removed from the 5′-En, can significantly stimulate replication.

MATERIALS AND METHODS

Construction of IgH-SV40 replicons. (i) pSVO, pOR1, pOR2, and pSEO. pEg1N was constructed by deletion of a core sequence from pIgHe, which contains the entire IgHe, a 0.9-kb *Xba*I fragment, in pUC19. The junction between 5′-En and 3′-En is a *Cla*I site. The SVO DNA fragment, containing the SV40 early promoter, enhancer, and ori (obtained from pSV_2gpt), was cloned into the *Acc*I site of pUC19 (pSVO). OR1 and OR2 inserts extended from the *Hin*dIII site (nucleotide 5171 in the SV40 numbering system) to the *Nco*I site (nucleotide 37) and to the *Sph*I site (nucleotide 128), respectively. These inserts were cloned into the *Acc*I site of pUC19 (pOR1 and pOR2). Seventy-two-base-pair repeats (enhancer) were inserted into pOR1 (pSEO). The orientation of SV40 sequences in SV40 plasmid vectors is indicated with a "+" or a "−" (direction of early mRNA starts identical or opposite to that of *lacZ* in pUC19, respectively).

(ii) pE-SVO, pE-OR1, pE-OR2, and pE-SEO. All SV40 fragments (SVO, OR1, OR2, and SEO) were excised and attached to *Cla*I linkers and cloned into the *Cla*I site of pEgIN (pE-SVO, pE-OR1, pE-OR2, and pE-SEO).

(iii) pESgpt and $pXSV_2gpt$. Flanking regions and SVO fragment-carrying DNA (E-SVO) were isolated from *Xba*I-digested pE-SVO. This fragment did not contain the polylinker region of pUC19. The SV40 early region of pSV_2gpt was replaced with the E-SVO fragment (pESgpt). The entire IgH enhancer was cloned into pSV_2gpt ($pXSV_2gpt$).

(iv) pKMH-OR1 and pKME-OR1. The MAR-containing segment [0.4-kb *Hin*dIII-*Hin*fI fragment of the Ig(κ) light chain] (KMAR) was isolated from pCk (41) and attached to *Eco*RI or *Hin*dIII linkers. Appropriately linked MAR DNAs were cloned into the *Eco*RI or *Hin*dIII sites, respectively, of pOR1+ (pKME-OR1 and pKMH-OR1).

(v) pOR1N, pOR1S, pE-OR1N, and pE-OR1S. To construct the pE-OR1 mutant such that the distance between 5′-En and 3′-En was equivalent to the IgHe core, a part of the bacterial *cat* gene was utilized as a spacer. A 240-bp fragment within the *cat* open reading frame was obtained from pSV_2CAT and was inserted into the 5′ or 3′ end of the OR1 fragment (in pOR1) to generate pOR1S and pOR1N (see Fig. 2E). The resulting OR1 *cat* fragments were then cloned into pEgIN (pE-OR1S and pE-OR1N [see Fig. 2E]).

(vi) p5′-OR1N and p3′-OR1N. 5′-En or 3′-En was deleted from pOR1N by the appropriate restriction enzyme digestion, and the plasmid was self ligated to generate p5′-OR1 and p3′-OR1N. For each IgHe-SV40 plasmid (except pE-OR2), two mutant clones which differed from each other only in the orientation of SV40 sequences were constructed.

Construction of the μE5 and 2 or E site-containing SV40 plasmids. Oligonucleotides corresponding to the IgHe μE5 and μE2 linked sites, 5′-CTAGAAGAACACCTG(μE5)-CAGC-AGCTGGCAGGT-3′ (μE2), or the E site, 5′-CTA GATAAATGAATTGAGCAATGTTGAGT-3′, were synthesized with *Xba*I sites at the ends. The double-stranded oligonucleotides were introduced into the *Xba*I site in pOR1, which is adjacent to the AT-rich region (ATR). To check the copy number of IgHe motifs and the orientation, DNA sequences were determined by the dideoxy-chain termination method (32).

Transient replication assay. Transfection of COS-1 cells was carried out by the DEAE-dextran method (25). COS-1 cells (2×10^5) were cotransfected with 100 ng of IgHe-SV40 and SV40 plasmid DNAs. At 48 h posttransfection, low-molecular-weight DNAs were extracted from the cells by the Hirt method (19), linearized by digestion with *Bam*HI, and cleaved with *Dpn*I to differentiate between the *Dpn*I-sensitive DNA and the *Dpn*I-resistant DNA (39). All the test plasmids (except p5′-OR1N) have a single *Bam*HI site within the polylinker region. p5′-OR1N has a single *Xba*I site. The digested DNAs were fractionated on 0.8% agarose gels, blotted onto nitrocellulose filters, and hybridized with a random-primed ^{32}P-labeled appropriate DNA fragment. The radioactivity of replicated DNA on the filter was quantified with a Betascope 603 Blot Analyzer (Betagen Corp.).

Gel retardation assays. Enhancer motif-containing, duplexed oligonucleotides were 5′ end labeled with T4 DNA kinase and used as probes. The preparation of nuclear extracts and gel retardation assays were performed as previously described (12, 23).

RESULTS

IgHe flanking region enhanced SV40 DNA replication in vivo. To test the possibility that IgHe regulates SV40 DNA replication, we first examined the effects of flanking sequences (5′-En and 3′-En [Fig. 1A]) for the following reasons: (i) the core region of IgHe contains SV40 enhancer core sequences (7, 14, 34), which appear to activate minimal origin replication, and (ii) flanking sequences have been demonstrated to suppress SV40 enhancer-mediated transcription in fibroblasts when the 5′-En and 3′-En are introduced into each end of the SV40 enhancer (20). We inserted the 5′-En and the 3′-En into the 5′ end and 3′ end, respectively, of the SVO fragment, which contains the ori, 21-bp repeats (promoter), and 72-bp repeats (enhancer). A schematic of this construction (pE-SVO) with different orientations of the SVO insert (+ and −) is shown in Fig. 1D. The replication efficiencies of the IgHe-SV40 test plasmids were measured by *Dpn*I-resistant and *Mbo*I-sensitive assays. After transfection of plasmid DNA into COS-1 cells, which constitutively express SV40 T antigen, the Hirt DNA obtained was cut with *Dpn*I to digest methylated input DNA or *Mbo*I to digest demethylated DNA and subjected to Southern analysis. In this assay, the *Dpn*I-resistant and *Mbo*I-sensitive band(s) corresponds to a newly replicated test plasmid. In the *Mbo*I assay, the residual input DNA (*Mbo*I-resistant bands in Fig. 1A) served as an internal control for the amount of plasmid DNA transfected. Results of two assays are shown in Fig. 1A. The flanking region enhanced SV40 DNA replication about threefold. The activity was not dependent on the orientation of SVO sequences located between 5′-En and 3′-En.

IgHe core sequence does not contribute to SV40 DNA replication. In order to test effects of the complete IgHe, the 0.9-kb *Xba*I fragment was introduced into pSV_2gpt (Fig. 1D). Activation of replication was indistinguishable, regardless of the presence of the core sequence (Fig. 1B). There-

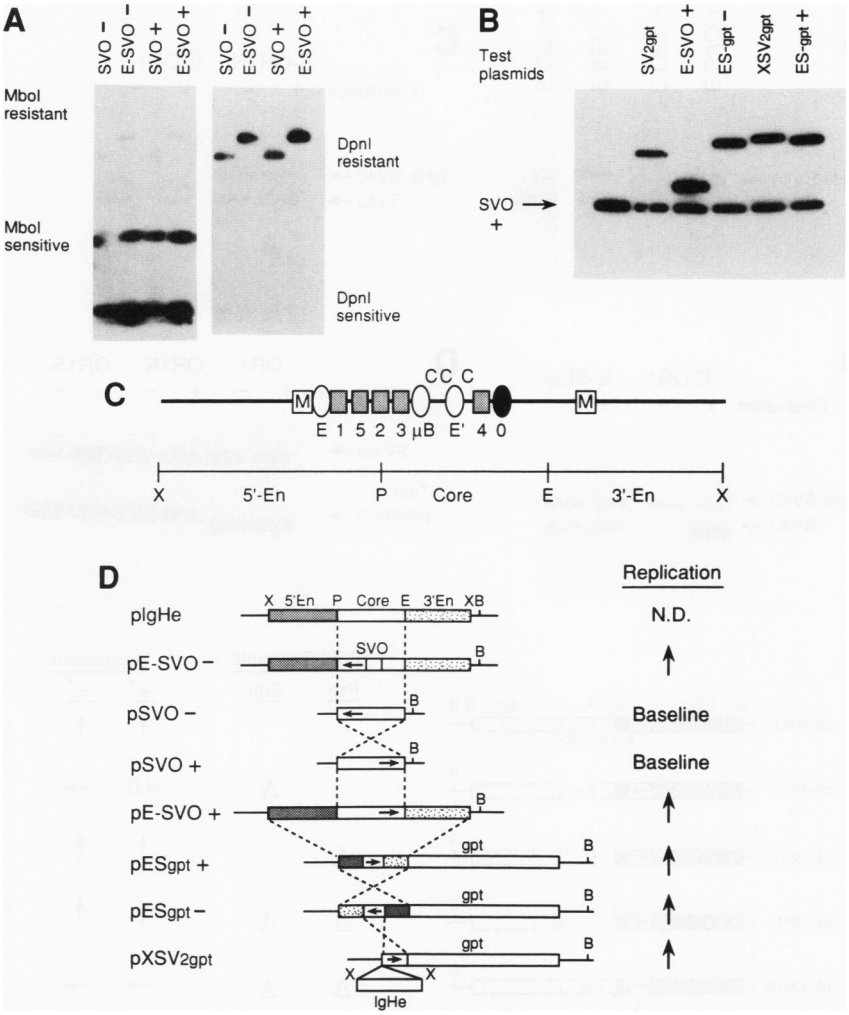

FIG. 1. Flanking regions of IgHe increase SV40 DNA replication. (A) IgHe-SV40 plasmids were individually transfected into COS-1 cells. Two days after transfection, low-molecular-weight DNAs (Hirt DNAs) were prepared from cells, digested with *Bam*HI and *Mbo*I (left panel) or *Bam*HI and *Dpn*I (right panel), and separated by 1% agarose gel electrophoresis. The DNAs were transferred to a nitrocellulose membrane and hybridized with a random-primed, ^{32}P-labeled SVO fragment. *Dpn*I-resistant and *Mbo*I-sensitive bands represent newly replicated DNAs. *Dpn*I-sensitive and *Mbo*I-resistant bands represent input DNAs. Test plasmids used are indicated at the top. (B) IgHe-SV4O plasmids were cotransfected into COS-1 cells with pSVO+ as a control. Hirt DNA was digested with *Bam*HI and *Dpn*I. Transient replication assays were performed as described above. The SVO fragment was used as a probe, which is identical among control and test plasmids. Test plasmids are indicated at the top. Upper bands represent linearized DNA for *Dpn*I-resistant test plasmids; lower bands represent linearized control plasmid pSVO+. To exclude the possibility of a polylinker effect on replication, we constructed pESgpt, which does not have the polylinker region (see panel D). pESgpt replicated more efficiently than control plasmid pSV$_2$gpt did, indicating that the polylinker was not responsible for the flanking-region effect on replication. (C) Schematic map showing the entire IgHe region, a 0.9-kb *Xba*I (X) fragment. IgH enhancer is operationally divided into three domains by *Pvu*II (P) and *Eco*RI (E): 5′-En, core, and 3′-En. Enhancer factor-binding sites are μE (E), μE1 (1), μE5 (5), μE2 (2), μE3 (3), μB, μE′ (E′), μE4 (4), and Oct (0). M, MAR; C, SV40 enhancer consensus sequence. (D) Maps of test plasmids showing the relationship between the flanking region of the IgHe and the SV40 DNA fragment. SV40 and IgHe DNA fragments were introduced into pUC19. For simplicity, the maps are from the 3′ end on the left to the 5′ end on the right except for pESgpt and pXSV$_2$gpt. Open bars, SV40, IgHe, and *gpt* gene segments. pUC19 is represented by a thin line. IgHe is the 0.9-kb *Xba*I fragment containing the complete IgHe. The shaded box represents the ATR in the SV40 replication origin. The arrow within the SV40 DNA fragment box indicates the direction of early mRNA starts. *Bam*HI, a unique site in most clones, was used to linearize the test plasmids. For p5′-OR1N, *Xba*I sites were used, because the *Bam*HI site was deleted during construction. + and − in the designations indicate the orientation of SV40 DNA as described in Materials and Methods. The results of transient replication assays shown above are summarized on the right. ↑, about threefold activation; baseline, activity equivalent to that of the control plasmid; N.D., not determined.

fore, it is unlikely that the core sequence has an effect on SV40 DNA replication.

Flanking regions also suppress SV40 DNA replication. The SV40 enhancer and promoter can stimulate their own DNA replication in *cis* (3, 24). To determine which regulatory element of SV40 is required for replicational activation by IgHe, each of the elements present on the SV40 fragment between 5′-En and 3′-En in pE-SV40 was deleted. pE-OR2, pE-SEO, and pE-OR1 have deletions of the enhancer, promoter, and both, respectively (see Fig. 2E). pOR2, pSEO, and pOR1 are the corresponding control plasmids that lack IgHe regions. Replication activity was determined by

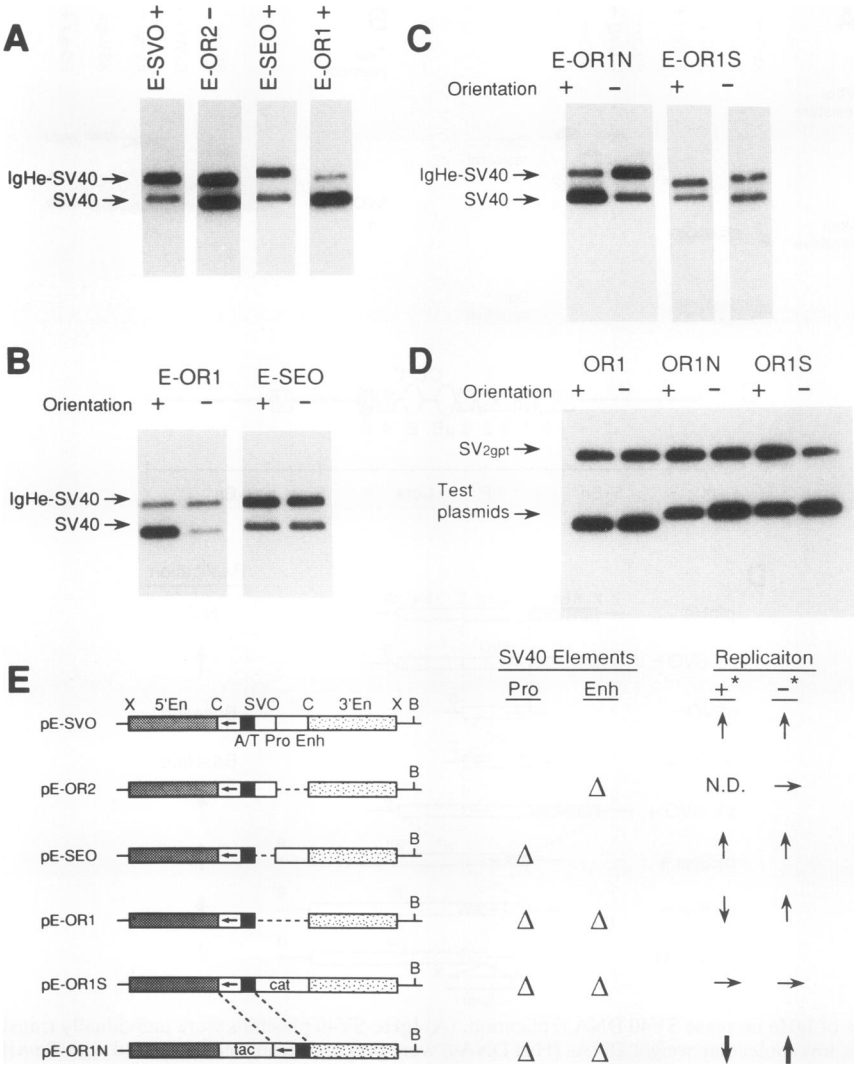

FIG. 2. Relative replication of deletion mutants of SV40. IgHe-SV40 plasmids were cotransfected into COS-1 cells with the corresponding SV40 control plasmids. DNAs were extracted 48 h after transfection and digested with *Bam*HI and *Dpn*I. Hybridization was performed with a ^{32}P-labeled SV40 fragment that was shared among control and test plasmids. The schematic maps of test plasmids are depicted in panel E. (A) Transient replication assays with IgHe-SV40 deletion mutants were performed. E-OR2, E-SEO, and E-OR1 have deletions of the enhancer, promoter, and enhancer-promoter, respectively. (B) Activity is dependent on the orientation (+ or −) of the SV40 ori fragment located between 5′-En and 3′-En of IgHe. (C) Effects of a spacer between 5′-En and 3′-En on IgHe-SV40 replication. The orientation (+ or −) of the SV40 ori fragment in each clone is indicated. Each test plasmid was cotransfected with a plasmid containing the SV40 ori fragment identical to that of the test plasmid. (D) pOR1 replication is unaffected by the orientation of SV40 or by introduction of the spacer. Replication activities of the indicated plasmids were compared with that of pSV2gpt as a control plasmid. (E) Summary of functional analyses of IgHe-SV40 plasmids with deletions of SV40 transcriptional elements and with spacer mutants. SV40 transcriptional elements were deleted from pE-SVO. The ATR is indicated (black box). Only the minus orientation of each replicon is shown here. cat, a truncated *cat* gene (see Materials and Methods); the order of the letters in "cat" corresponds to the direction of *cat* mRNA starts. Deletion of the SV40 promoter (Pro) or enhancer (Enh) region is indicated by a horizontal dashed line and Δ. Replication activities, relative to the corresponding control lacking IgHe sequences, are summarized for the SV40 orientations (+ and −) of each clone. Thick vertical arrows, approximately fivefold effects; thin vertical arrows, approximately threefold effects; horizontal arrows, no effect.

cotransfection. The IgHe flanking region activated pSVO and pSEO replication threefold but had no effect on pOR2 (Fig. 2A). Surprisingly, pOR1 DNA replication was suppressed fivefold. Although these experiments did not reveal which SV40 regulatory element was being targeted, the results suggested that the mechanism of action of flanking regions is complex. Two potentially contributing factors, polarity and steric hindrance, are addressed in sections below.

Suppressive effect of flanking regions on the SV40 origin (OR1) is dependent on the orientation of the SV40 ori sequence. It is possible that the activity of flanking regions is dependent on the orientation of the SV40 regulatory element(s) inserted between flanking regions. SV40 deletion mutants (with the exception of pE-OR2+) were constructed in opposite orientations to test for a polar effect. We could not construct pE-OR2+; presumably, it was lethal to *Escherichia coli* because all 36 positive clones screened had a

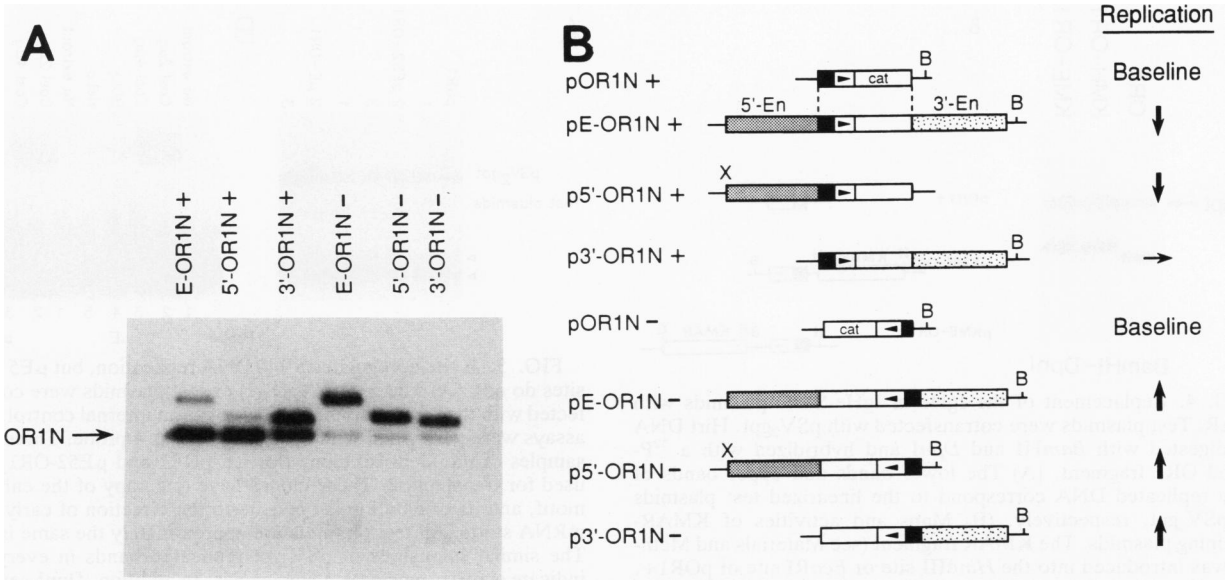

FIG. 3. Effect of 5′-En and/or 3′-En of the IgHe on SV40 DNA replication. (A) Test plasmids were cotransfected with pOR1N+ or pOR1N−, extracted, and digested with *Bam*HI and *Dpn*I. p5′-OR1N+ and p5′-OR1N− were digested with *Xba*I and *Dpn*I. Upper bands correspond to the linearized test plasmid. Test plasmids are indicated at the top. All test plasmids contained identical origins of SV40. An OR1 *cat* fragment was used as the hybridization probe. (B) Maps of deletion mutants of the IgHe domain. Activities are indicated relative to that of pOR1N. See Fig. 1 and 2 legends for symbols and abbreviations.

"minus" orientation. Figure 2B shows that a strong polarity effect was exerted on the origin (pE-OR1), but suppression was not observed when the ori and IgHe flanking sequence were separated by the SV40 enhancer (pE-SEO) or by the enhancer plus the promoter (pE-SVO). The level of suppression by the flanking region on OR1 replication was significantly greater than the activation by flanking regions (Fig. 2B; compare E-OR1+ and E-OR1−). The question of whether the control plasmid (pOR1) for pE-OR1 expressed different activities depending on the orientation of the SV40 ori sequence arose. To address the issue, pOR1+ and pOR1− were cotransfected with pSV_2gpt as a control. The data of Fig. 2D indicated that the replicative activity of pOR1 was constant regardless of the orientation.

Steric hindrance does not contribute to the flanking-region effect. In pE-SVO, the 5′-En and 3′-En are separated by 344 bp. However, the deletion mutants in Fig. 2 have shorter distances: pOR2, pSEO, and pOR1 are 200, 301, and 104 bp, respectively. To examine the possibility that there is steric hindrance of SV40 DNA replication by flanking regions, two types of spacer mutants were constructed. A truncated *cat* gene was excised as a 240-bp *Hin*dIII-*Eco*RI fragment from pSV_2cat. The fragment was derived from the *cat* ORF, thus satisfying the criterion that it not be a regulatory element. When this spacer was inserted into pE-OR1, the separation corresponded to the distance between flanking regions in pE-SVO. The spacer is approximately the same length as the SV40 enhancer and promoter deleted from pE-OR1. When the truncated *cat* gene fragment was introduced at a location adjacent to the ATR (pE-OR1S), it had no effect (Fig. 2C). When *cat* was inserted downstream of the early mRNA starts (pOR1N) of the ori (Fig. 2E), negative and positive effects, depending on the orientation of the SV40 ori with respect to flanking regions, were seen (Fig. 2C). The level of enhancement or suppression observed was approximately the same, and the effect of IgHe on pOR1N is consistent with that of pOR1. Moreover, as shown in Fig. 2D, both spacer mutants replicate at levels similar to that of pOR1, independently of the orientation of the ori sequence.

Experiments presented in the preceding sections collectively indicate that the flanking regions interact with the ori sequence to regulate DNA replication (Fig. 2E). Either up- or downregulation can be achieved, and it is dependent upon the relative orientation of the origin. Neither steric hindrance nor other SV40 regulatory elements (enhancer or promoter) are required to achieve the effect.

Flanking-region activity is dependent on its position relative to the AT-rich region of the SV40 ori sequence. The above experiments tested the effect of IgHe flanking regions on replication using test plasmids containing both 5′-En and 3′-En. Next, functional analyses of 5′-En and 3′-En were performed independently to determine whether both flanking regions are required. The pOR1N vector (Fig. 3) was used as a control to simplify interpretations. That is, opposite orientations of pOR1N activated or suppressed replication to similar levels as opposed to pOR1, which is subject to polar effects. The 5′-En or 3′-En was removed from pE-OR1N, and two sets of test plasmids were constructed (Fig. 3). 5′-OR1N and 3′-OR1N are of similar sizes and about 300 bp larger than the pOR1N control, allowing us to distinguish test plasmids and control plasmids in an agarose gel. As shown in Fig. 3A, 5′-OR1N+, in which the 3′-En is deleted, showed activity equivalent to that of the wild type (pE-OR1N+) while deletion of the 5′-En (3′-OR1N+) resulted in no activity. Therefore, the 5′-En is required for the orientation-sensitive suppression expressed by the wild type. However, when the 5′-En was positioned at a distance from the ATR (E-OR1N− and 5′-OR1N− in Fig. 3), the 5′-En activated pOR1N replication to the same extent as the 3′-En. When the 3′-En was inserted in proximity to the ATR it worked as an activator. On the other hand, the 3′-En had no effect on replication when positioned at a distance from the ATR. Therefore, independent activities of the 5′-En and 3′-En depend on their positions with respect to the ATR of

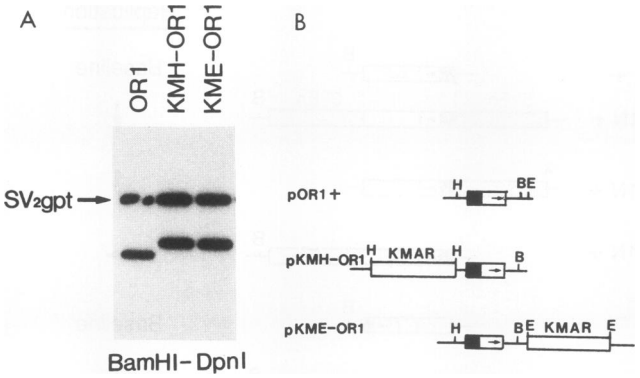

FIG. 4. Replacement of the IgHe in IgHe-SV40 plasmids with KMAR. Test plasmids were cotransfected with pSV_2gpt. Hirt DNA was digested with *Bam*HI and *Dpn*I and hybridized with a ^{32}P-labeled OR1 fragment. (A) The lower bands and upper bands of newly replicated DNA correspond to the linearized test plasmids and pSV_2gpt, respectively. (B) Maps and activities of KMAR-containing plasmids. The KMAR fragment (see Materials and Methods) was introduced into the *Hin*dIII site or *Eco*RI site of pOR1+. The maps show the structural relationship between KMAR and SV40 ori.

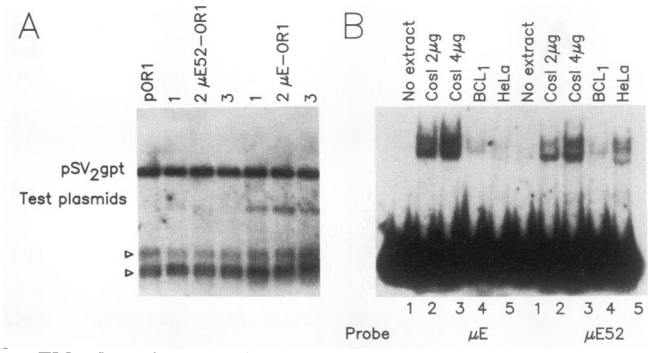

FIG. 5. E site upregulates SV40 DNA replication, but μE5 and -2 sites do not. (A) The control (pOR1) or test plasmids were cotransfected with the same amount of pSV_2gpt as an internal control. *Dpn*I assays were performed with SVO fragment as a probe. Three DNA samples of the identical clone (for μE-pOR1 and μE52-OR1) were used for transfection. These clones have one copy of the enhancer motif, and its orientation is opposite to the direction of early SV40 mRNA starts. All test plasmids are approximately the same in size. The similar intensities of pSV_2gpt replicated bands in every lane indicate similar transfection efficiencies. In addition, *Dpn*I-sensitive upper and lower bands (triangles) represent unreplicated DNAs of pSV_2gpt and pOR1 vectors, respectively, and serve as another internal control to show the same amount of input DNA. (B) The oligonucleotides containing the E site or the μE5 and -2 sites were 5′ end labeled with ^{32}P and used as electrophoretic mobility shift assay probes. Three micrograms each of BCL_1 and HeLa nuclear extracts were used.

SV40 ori. The equivalent intensities of bands (Fig. 3, "minus" test plasmids) suggest that the 5′-En and 3′-En did not synergize.

Flanking-region activity is not dependent on MAR function. Both the 5′-En and the 3′-En fragments used here have been shown to bind the nuclear matrix in vitro (9) and contain consensus topoisomerase II sites. DNA replication has been suggested to initiate at or close to MARs in chromosomal DNA (42). KMAR has a higher in vitro affinity for the nuclear matrix than either the 5′-En or the 3′-En (8). To investigate the possibility of MAR function in our system, flanking regions in pE-OR1 were replaced with KMAR. KMAR was inserted downstream of the early mRNA starts (pKMH-OR1) or proximally to the ATR region (pKME-OR1) of the OR1 fragment (Fig. 4). Figure 4 shows that addition of the higher-affinity MAR made little difference. Therefore, it is unlikely that the IgHe flanking region effect is mediated through MARs.

The E site upregulates SV40 DNA replication. Several nuclear factor-binding sites in the 5′-En and core sequences have been identified (Fig. 1C), whereas little is known about factor-binding sites in the 3′-En. We focused on the 5′-En and examined whether a characterized site could influence SV40 DNA replication. The 5′-En *Xba*I-*Pvu*II fragment contains, in 5′-to-3′ order, E, μE1, μE2, and μE5 motifs (reviewed in reference 37). With the exception of μE1, these sites have been shown to bind well characterized, ubiquitously expressed nuclear proteins (37). Recently, one of the CCAAT-binding nuclear factors, NF-I/CTF, has been shown to stimulate SV40 DNA replication (6). One of the E-site-binding factors, Ig/EBP-1 (Fig. 1C), has been identified as a member of the family of CCAAT-binding factors (31). Therefore, we reasoned that the E-site interaction might contribute to the regulation of SV40 DNA replication by the 5′-En.

To evaluate the above hypothesis, we inserted an E site or linked μE5 and μE2 sites at a location adjacent to the ATR of the SV40 minimal origin (pOR1). The μE5 and μE2 sites were placed on the same oligonucleotide, since they are separated by just 4 bases in wild-type IgHe. Each of the three clones of each construct had one copy of the oligonucleotide whose orientation is opposite to the direction of early mRNA. These test plasmids were cotransfected with pSV_2gpt as a control. Transient replication assays (Fig. 5A) indicated that the E-site-containing SV40 plasmids (μE1-OR1) replicated at significantly higher levels than pOR1 whereas the μE5- and μE2-site-containing plasmids (μE52-OR1) replicated at similar levels.

The failure of μE5 and μE2 sites to enhance SV40 DNA replication would be expected if the corresponding nuclear binding factors were absent or present at low levels in COS1 cells. To the contrary, gel retardation assays confirmed that COS-1 cells contained E-, μE5-, and μE2-binding activities at levels higher than those in a B-cell line, BCL_1 (Fig. 5B). Competition assays showed that the lowest band in COS-1 nuclear extracts is due to nonspecific binding of the probe (data not shown). These experiments suggest that an E-site-binding factor(s) contributes to upregulation by the 5′-En.

DISCUSSION

In a previous study (1), we mapped a chromosomal origin which is located very close to the IgH enhancer region and appeared to be regulated by transcription. On the basis of these observations, we hypothesized that the IgH enhancer contributed to the regulation. In view of the technical difficulties associated with a direct evaluation of this hypothesis at the chromosomal level, we have utilized the origin of SV40 DNA replication as a model of the IgH chromosomal origin. Results presented here strongly suggest that the flanking domains of the IgH enhancer can directly regulate DNA replication in lieu of interactions with the SV40 enhancer or promoter. Moreover, the study indicated that the effect is due at least in part to E site binding. The mechanism appears complex, as indicated by the observation that the influence of the enhancer flanking regions is dependent upon

their positions and orientations relative to the ATR of the SV40 ori sequence.

IgHe flanking regions interact with the SV40 ori sequence to influence DNA replication with one exception: no effect is observed in the presence of the SV40 promoter (e.g., on pOR2). The magnitude of the stimulation by promoters has been shown to be much greater than that by enhancers (6, 24). ori enhancer plasmid pSEO replicate less effectively than ori promoter plasmid pOR2. pOR2 appeared to replicate at the maximal level. Therefore, we concluded that flanking regions failed to enhance ori promoter pOR2. This interpretation is supported by the result that flanking regions did enhance ori promoter-enhancer pSVO, since pSVO replicates less efficiently than pOR2 in our system. Promoters and enhancers seem to be inhibitory to stimulation of DNA replication mutually rather than synergistically (24). The greatest magnitude of stimulation by flanking regions was achieved when they were linked to the minimal ori sequence (pOR1). This observation implies that the mechanism of flanking-region activity is similar to those of SV40 promoters and enhancers.

When the 5′-En was placed at a distance from the ATR of the origin, DNA replication was enhanced. But when it was adjacent to the ATR, DNA replication was suppressed. The 3′-En was also found to enhance DNA replication when it was proximal to the ATR. The relationship between replication and the position of the 3′-En can be rationalized by a consideration of the stimulation of replication by SV40 regulatory elements (3, 24). The SV40 enhancer and promoter elements are capable of stimulating DNA replication only when these elements are inserted proximally to the ATR. The function of the 5′-En is more complex and may be dependent on the steric alignment, including orientation, rather than the position relative to the ATR.

It is unlikely that MARs play any role in the activation of SV40 DNA replication by flanking regions. This implies that the activation we have observed is independent of the topological location of the DNA replication apparatus but dependent on the functions of enhancer factors. However, SV40 DNA may need to associate with the nuclear matrix for replicating DNA (29), since DNA polymerase α (36), DNA primase (43), and topoisomerase II (4), which are involved in DNA replication, are present in the nuclear matrix. T antigen has been demonstrated to bind directly to purified DNA polymerase α (13). Conceivably, T antigen could be a link between the DNA template and the nuclear matrix, providing the equivalent of a MAR effect.

We found that the E site upregulated SV40 DNA replication but μE5 and -2 sites did not. The failure of the μE5 and -2 sites did not correlate with the absence of their binding factors in COS-1 cells. However, this upregulation is inconsistent with the effect of 5′-En in terms of the relationship between influence on replication and location of 5′-En; i.e., the 5′-En, when proximal to the ATR, suppressed replication, while the E site, when proximal to ATR, activated it. The relative orientations between the ATR and the E site in the p5′En-OR1N- and μE-OR1 constructs are opposite. However, the relationship between the relative orientations of the ATR and E sites and the effect is consistent, since both p5′-OR1N+ and μE-OR1 have the same relative orientation and both activate replication (Fig. 3). The mechanism of the 5′-En stimulation could be complex, since it is dependent on both orientation and position. At minimum, our data suggest that at least one class of IgHe factors are capable of regulating DNA replication. Any or several of the five E-site-binding, C/EBP family members might be involved. However, the best candidate is Ig/EBP, which is expressed at high levels in both COS-1 and early B cells (31). There are no E sites within the 3′-En which can exert effects in the absence of the 5′-En. Therefore, at least one additional *cis*-acting element is capable of participating.

Binding to μE5 and μNR motifs within the IgHe flanking regions has been interpreted as suppression of the activity of the core sequence of the IgH enhancer (33, 37). Our results provide an additional and/or alternative mechanism for flanking region-mediated repression of IgH transcription. In non-B cells, binding to flanking region motifs such as the E site leads to an interaction with the IgH-associated origin that downregulates IgH DNA replication. A consequence of this interaction is suppression of IgH transcription.

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