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An Enhancer of Recombination in Polyomavirus DNA

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Previous work from this laboratory has indicated that intramolecular homologous recombination of polyomavirus (Py) DNA is dependent upon promoter structure or function. In this report, we demonstrate that Py DNA contains not two but three binding sites for transcription factor YY1, all located on the late side of viral origin of replication (*ori*) and the third well within the VP1 coding sequence. This third site (Y3), which may or may not play a role in transcription regulation, is immediately adjacent to a previously described recombination hot spot (S_1/S_2). We found that Py replicons carrying an altered Y3 site recombined in a manner suggesting partial inactivation of the S_1/S_2 hot spot. Point mutations precluding the binding of YY1 to Y3 in vitro depressed hot spot activity in vivo; however, of the two reciprocal products reflecting recombination at this spot, only that carrying the mutated Y3 site arose at a reduced rate. These results are interpreted in light of a model assuming that recombination occurs within a transcriptionally active viral chromatin tethered to the nuclear matrix by YY1.

Most models of general recombination view this phenomenon as an interaction between two homologous DNA duplexes that have undergone pairing via an unspecified process (22, 33, 59). In higher organisms, genes exist in the form of chromatin, and it is therefore unclear how the proteins involved in cutting, transferring, and rejoining DNA strands gain access to their target. If this problem is real, it may be particularly serious for genes buried in inactive chromatin, and this may explain why transcription enhances recombination in *Saccharomyces cerevisiae* (25, 46, 56, 60, 64) and in mammalian cells (40). Actually, the observed correlation between transcription and recombination can be interpreted in two ways. The first interpretation is that the type of chromatin structure that allows—or results from—transcription could favor recombination. However, such an interpretation implies that recombination occurs almost fortuitously in eukaryotes and via a mechanism radically different from that operating in prokaryotes. Indeed, transcription-driven recombination has also been observed in prokaryotes (12, 24), even though in prokaryotes, transcription could alter DNA, but not chromatin structure. A second interpretation assumes the existence of a mechanistic link between transcription and recombination. For instance, transcription could generate positive supercoils, which would favor recombination (12, 16, 29, 65), or displace one strand from one of two homologous duplexes and allow this strand to invade the other duplex (25, 64) or bring to the site of recombination a protein needed for not only transcription elongation but also recombination, such as a topoisomerase (60). In all these conditions, transcription initiation as well as transcription elongation would be required for stimulation of recombination.

In a study carried out with polyomavirus (Py) DNA, we have obtained data compatible with intramolecular homologous recombination being transcription dependent. First, we noted that small deletions or substitutions within the viral early promoter appeared to significantly depress recombination between two sites distal to this promoter; similarly, inversion of the viral late promoter appeared to depress recombination between the same two sites (39) (see also Fig. 1). In these initial experiments, the recombination template could give rise to only one amplifiable product, while simultaneous detection of template and product required that both be amplified by autonomous replication (39). It was thus conceivable that rather than recombination itself, the mutation introduced into the promoter simply had affected detectability of the product more than that of the template. Therefore, in subsequent experiments, we made use of several modified constructs that were able to yield several alternative amplifiable products from any given substrate or template molecule (38). We then showed that, for a given combination of promoter mutations, recombination would give rise to some products but not to others, control experiments indicating that in every instance, product formation but not product detectability was the cause (38).

To further understand the dependence of Py DNA recombination upon promoter structure or function, we recently turned our attention toward transcription factors. Uniquely interesting among these factors is YY1 (51, 52, 54), also designated δ or NF-E1 elsewhere (18, 41), a highly conserved (18, 41) zinc finger protein (52, 54) which is able to initiate, activate, or repress transcription (for a review, see reference 53). YY1 is believed to regulate transcription by primarily affecting promoter structure, so that inverting a YY1 site in a given promoter may change this site from a negative to a positive regulatory element (37). In the adenovirus system, inhibition of transcription initiation by YY1 has been shown to be relieved by the viral E1A proteins (52) which interact specifically with YY1 in vitro (28). In the case of Py DNA, the transcriptional enhancer (61, 63) was shown to carry a binding site for a factor initially designated NF-D (9), later found to be identical or closely similar to YY1 (8a). The same site in the enhancer, which we shall designate Y1 here, was described by others as binding a factor called BF-H, which also bound to a second site (Y2) distant from Y1 by about 25 bp (20). We show here that YY1 binds not only to Y1 and Y2 but also to a third YY1 binding site (Y3) present in Py DNA. It is at present unclear whether and how Y3 would play a role in transcription

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regulation. However, our data clearly indicate that point mutations which abolish binding of YY1 to Y3 in vitro strongly depress recombination at a proximal site, while having no effect on recombination at distal sites. The fact that the observed reduction in recombination frequency concerns only one of two reciprocal products is furthermore consistent with our model of transcription-dependent, nonconservative, homologous recombination (38).

MATERIALS AND METHODS

Cells and DNAs. The origins of the mouse 3T6 cells and *ts*P155 mutant of Py have been described elsewhere (5, 6). RmI, originally cloned at the *Sal*I site of pAT153 (42), has been recloned at the same site in pNN, a vector derived from pAT153 (see below). Prior to transfection of 3T6 cells (57), each recombinant molecule carrying RmI or one of its mutants was cleaved with *Sal*I, and the insert was circularized by ligase treatment. Transfected cells were incubated for 3, 5, or 6 days at 33°C before DNA was extracted by the method of Hirt (21) and digested with *Dpn*I (which converts the input, methylated DNA into small fragments) and, generally, a restriction enzyme which linearizes both the recombination substrate and its product(s). After electrophoresis through 1% agarose gels, blotting, and hybridization with a radioactive viral DNA probe (55), samples were autoradiographed with Amersham Hyperfilm-MP and intensifying screens (Cronex Par Speed; Dupont). To ease our work, we created a new vector lacking a number of unique restriction sites also present in Py DNA. With the help of PCR (48), a DNA fragment spanning bp 574 to 4347 in pAT153 was synthesized by using this plasmid DNA as the template and NN1 and NN2 as primers (see "Oligonucleotides" below). After purification, this fragment was digested with *Cla*I (NN1 and NN2 both carry a *Cla*I site) and self-ligated. The resulting vector, pNN, was propagated in *Escherichia coli.*

Point mutants. Mutations in the Py late coding region of RmI (either in or near Y3) were generated by the PCR-mediated mutagenesis method of Barettino et al. (3) with pNN RmI (RmI cloned at the *Sal*I site of pNN) as the sole substrate. Briefly, DNA fragments with partly overlapping sequences (*x* and *y*) were synthesized in the first stage. Fragment *x* extended from Ins (bp −669) to the viral late coding region (bp 3305) of RmI (Fig. 1), with the desired mutations incorporated into the primer complementary to the viral DNA (see "Oligonucleotides" below). This specific fragment was mixed with another PCR-synthesized fragment, *y*, which was exclusively viral and common to all constructs and extended from bp 3289 to 4649. Taking advantage of the overlap between fragments *x* and *y* (bp 3289 to 3305), a third fragment, *z*, was synthesized in the second stage. This PCR fragment included the portion of RmI extending between the unique *Bgl*II site and the unique *Eco*RV site and encompassing the Y3 site as well as repeat S_2 (Fig. 1; see also Fig. 4A). Note that the portion of fragment *x* which included the mutations (bp 3270 to 3286 [see Fig. 4A]) was not part of overlap with fragment *y*. Following digestion with *Bgl*II and *Eco*RV, the trimmed *z* fragment was exchanged with its homolog in pNN-cloned RmI (Fig. 1). From these recombinant plasmids each carrying a different mutant RmI as insert, other plasmids carrying mutant O/RmI molecules (38) were constructed. Thus, a fragment of cloned Py DNA encompassing the regulatory region (bp 4632 to 1192) obtained by partial digestion with *Sau*3A was inserted at the *Bgl*II site of RmI (Fig. 1; see also Fig. 9A).

Deletion mutants. Y1 and Y2 were deleted from Py DNA making use of PCR and of appropriate primers (see "Oligonucleotides" below), as described elsewhere (39). Primers flanking Y1, ΔAma 1 and ΔAma 2, were used in combination with Kp2 and Kp1, respectively, thus generating two fragments with a *Mlu*I site at one end and a *Kpn*I site at the other end. These two fragments were digested with *Mlu*I and ligated to one another, thus giving rise to a larger fragment extending from bp 4686 to 2188 through the viral origin and carrying a *Mlu*I site (5′-ACGCGT-3′) instead of site Y1 (5′-CGCCATCTT-3′; see Fig. 2A). This fragment was trimmed with *Kpn*I and substituted for its homolog in pNNRmI. The same strategy was used with primers ΔHir1 and ΔHir2 to delete Y2.

EMSA. Nuclear extracts from mouse 3T6 cells were prepared by the method of Andrews and Faller (1). Following extraction, the protein concentration was determined with the Bio-Rad protein assay by the method of Bradford (8). Binding reactions were carried out in a 20-μl reaction mixture containing 12 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-KOH (pH 7.9), 168 mM NaCl, 12% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of bovine serum albumin, 2 μg of poly(dI)-poly(dC) as well as 6 μl of crude nuclear extract (approximately 15 μg of total protein) or 200 to 400 ng of purified glutathione *S*-transferase (GST)-YY1 (see below). The mixture was incubated at room temperature for 5 to 10 min before and 30 min after addition of the radioactive probe (0.2 ng of ^{32}P-labelled DNA; ca. 20,000 cpm) and then loaded on a 4% acrylamide gel (acrylamide-bisacrylamide [40:1]) that had been prerun at 100 V for 30 min in 0.5× TBE (Tris-borate-EDTA) buffer. The loaded gel was run at 150 V (10 V/cm) for 90 to 120 min, vacuum dried on Whatman 3MM paper, and placed on a Hyperfilm-MP (Amersham). The film was exposed for 3 h or overnight at −80°C. For anti-YY1 supershift assays, 1 or 5 μl of a human monoclonal antibody directed against YY1 (kindly provided by T. Shenk) was added to the electrophoretic mobility shift assay (EMSA) mixture after the 30-min reaction time, and the incubation was continued for 1 h at 4°C before the samples were processed as described above. For competition experiments, we used as unlabelled competitor DNA a double-stranded oligonucleotide containing the YY1 binding sequence from the P5+1 element of the P5 promoter from adeno-associated virus type 2 (52). This DNA was prepared by annealing two single-stranded oligonucleotides, YY1a and YY1b (see "Oligonucleotides" below), as described in "DNA bending" below for Bend I and Bend II. The YY1 oligonucleotide was incubated with nuclear extracts for 20 min at room temperature prior to the addition of the radioactive probe. DNA fragments (80 to 140 bp long) used as probes—and sometimes as competitor DNA—were synthesized by PCR with several different primers (see "Oligonucleotides" below and see Fig. 2 and 4). 3′-end labelling was done with terminal transferase and [α-^{32}P]ddATP (68), before the fragments were purified on Sephadex G50 (Nick columns from Pharmacia).

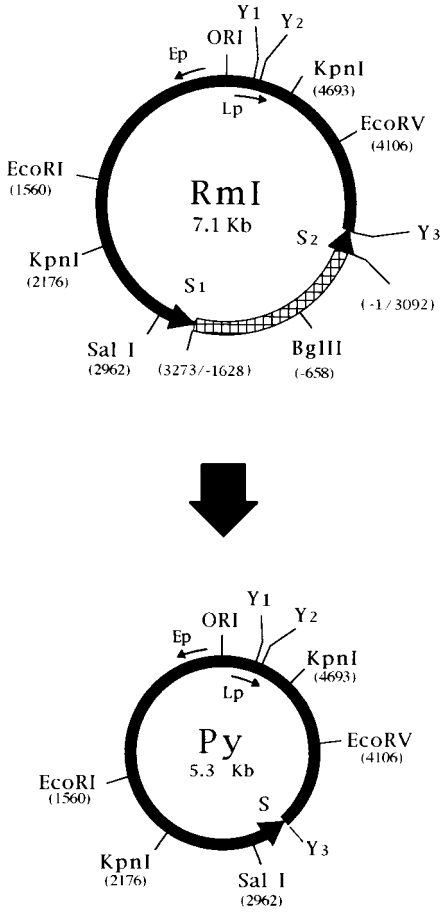

FIG. 1. Substrate and product of recombination. RmI is a circular molecule consisting of 1,628 bp (negative numbering) of mouse DNA (Ins [checkered box]) inserted in the late-coding region of a *ts*P155 Py genome (thick line), 182 bp of which are directly repeated (repeats S_1 and S_2 [large arrowheads]). RmI thus includes one complete, uninterrupted viral early coding region and is converted into unit-length viral DNA (Py DNA) via homologous recombination between S_1 and S_2. The viral regulatory region includes the origin of replication (ORI) and two opposing promoters, early (Ep) and late (Lp). Also shown on the figure are YY1 binding sites (Y1, Y2, and Y3 [see text]).

DNA bending. Equal molar amounts of oligonucleotides Bend I and Bend II (see "Oligonucleotides" below) were mixed in a solution containing 40 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, and 22 mM dithiothreitol and annealed by being boiled for 3 min and then slowly cooled to room temperature. The resulting double-stranded oligonucleotide (bp 3265 to 3293), which included Y3 (bp 3270 to 3278) and termini compatible with a *Sal*I end and a *Xba*I end, was inserted into similarly digested plasmid pBend2 (kindly provided by S. Adhya; 26). Fragments generated by a series of restriction endonucleases and containing Y3 at various positions with respect to the ends (see Fig. 5A) were 3′ end labelled, purified, and subjected to EMSA as described above.

Purification of GST-YY1 fusion protein. The pGST-YY1 plasmid, harboring the entire YY1 coding region in frame with the GST gene, was generously provided by T. Shenk as a transformed *E. coli* DH5α strain. GST-YY1 was

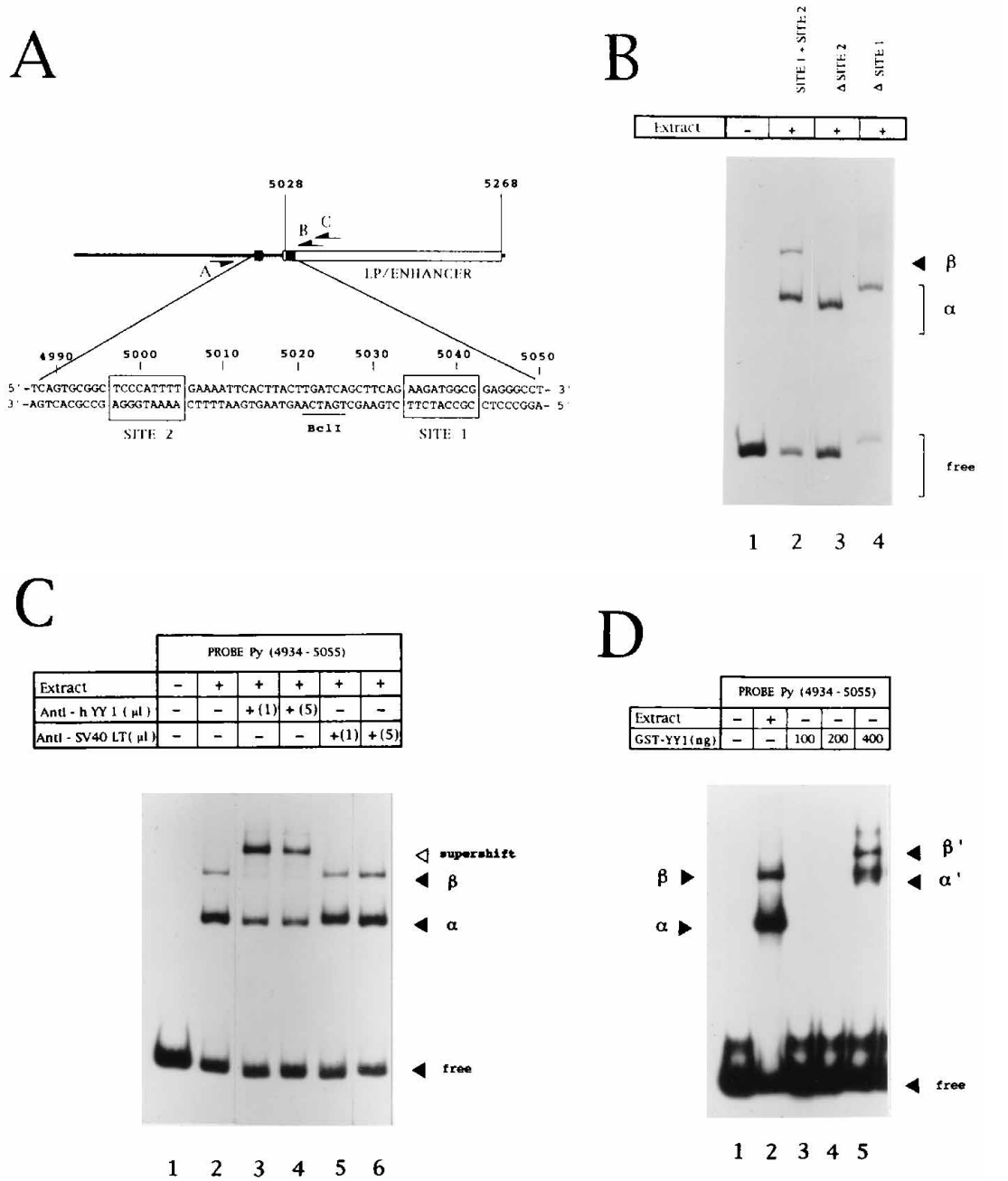

FIG. 2. Two YY1 binding sites flank the viral *Bcl*I site at bp 5021. (A) Map of the region. The top drawing shows the late promoter (LP)-enhancer region (bp 5028 to 5268 [see references 61, 63, and 67]) as a white box and the two YY1 sites (20; this work) as small black boxes. Shown below is the sequence of the DNA including Y1 (site 1) and Y2 (site 2) (these two sites boxed as done previously [20]). Half arrows indicate primers used to PCR amplify the DNA subjected to EMSA (A, ERL4; B, PB1; C, PBX [see Materials and Methods]). (B) EMSA on fragments synthesized by using the primers indicated above and either full-length RmI (lanes 1 and 2), RmI lacking Y2 (lane 3), or RmI lacking Y1 (lane 4) as the DNA template. The A-C rather than A-B combination of primers (see panel A) was used for synthesis of the fragment run in lane 4, making this fragment larger (see also Materials and Methods). Mouse 3T6 extract was incubated (+) or not incubated (−) with the DNA fragments. Note also that only full-length DNA gives rise to two complexes, α and β (lane 2). The position of uncomplexed DNA (free) is also shown. (C) DNA amplified from full-length RmI (with primers A and B) was incubated with mouse 3T6 extract (lanes 2 to 6) and two different amounts of anti-hYY1 (lanes 3 and 4) or anti-SV40 large T (lanes 5 and 6) antibodies, prior to EMSA. Note with the anti-hYY1 antibody, the appearance of a third band (supershift), correlating with a strong reduction in the amounts of the α and β complexes. (D) The DNA fragment used in panel C was subjected to EMSA after being incubated with either no protein (lane 1), mouse 3T6 extract (lane 2), or increasing amounts of GST-YY1 protein (lanes 3 to 5). Complexes obtained with the GST-YY1 protein and designated α′ and β′ here (lane 5), are presumed to be slower migrating than the α and β complexes, because GST-YY1 is larger than YY1 itself.

produced and purified as follows. A 1-liter bacterial cell culture was grown to 0.6 optical density unit and induced with 0.1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 4 h. The cells were then pelleted and lysed in a solution containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 1 mM EDTA. Insoluble debris were removed by centrifugation at 25,000 × *g* for 30 min. Expression of the GST-YY1 fusion protein in the supernatant was monitored either by a GST-specific photometric assay (conjugation of glutathione with 1-chloro-2,4-dinitrobenzene; Pharmacia) or by immunoprecipitation, using either anti-GST antibody (Pharmacia) or anti-YY1 antibody (provided by T. Shenk). GST-YY1 protein was purified by affinity chromatography on a glutathione-Sepharose

column (Pharmacia). After the column was washed with 10 bed volumes of phosphate-buffered saline A, the fusion protein was eluted with a buffer containing 50 mM glutathione and 500 mM NaCl. The GST-YY1 protein was about 80% pure, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining (see Fig. 3B).

YY1 expression vectors. Mouse 3T6 cells were cotransfected with RmI or O/RmI and one of four recombinant plasmids constructed with the help of pCMV YY1 (31, 44) which was kindly provided by Yang Shi. The first two of these plasmids, pPyOriCMV YY1(+) and pPyOriCMV YY1(−), were constructed by inserting a segment of Py DNA encompassing the origin of replication (bp 5157 to 83) into the *Xba*I site located upstream of the cytomegalovirus promoter in pCMV YY1. The Py DNA had been PCR synthesized by using two primers carrying an *Xba*I site toward their 5′ ends, DG1 and DG2. The construction of the second set of plasmids involved excising the cDNA for YY1 from pCMV YY1 and cloning it at the *Bam*HI site of the multiple cloning site region of pcDNAI/Amp, a commercially available (Invitrogen) vector already including a Py DNA origin. The two resulting plasmids were designated pYY1 cDNAI/Amp(+) and pYY1 cDNAI/Amp(−). All four plasmids derived from pCMV YY1 were tested for autonomous replication in mouse 3T6 cells and in *Scop*-T1 cells, a line of mouse cells constitutively expressing Py early proteins (43). Southern transfers of low-molecular-weight DNAs from the transfected cells indicated that all four plasmids replicated readily in *Scop*-T1 cells but not in 3T6 cells, as expected if Py early proteins had been required for such replication. Over a 5-day period, accumulation of the plasmids derived from the Invitrogen vector, however, was about three to five times greater than that of the two other plasmids.

Oligonucleotides. All primers were synthesized with a Pharmacia Gene Assembler Plus. In the sequences shown below, homology with the template DNA is specified in two ways: homologous nucleotides are underlined, while the numbers in parentheses specify the coordinates (in base pairs) on the template DNA. Nucleotides forming a restriction site mentioned in this study are shown in italic type. The oligonucleotide primers used in the different processes are as follows: (i) construction of pNN, NN1 (574 to 590), 5′-TAG*ATCGAT*CCTTGCGGCGGCGGTGC-3′; and NN2 (4347 to 4331), 5′-TAG*ATCGAT*GGGCCTCGTGATACGCC-3′; (ii) construction of point mutations, MUT1 (3305 to 3261), 5′-ACACCCTGACAACTGTGCTCCTAGATGAGAACGGCGTGGGGCCCC-3′; MUT2 (3305 to 3264), 5′-ACACCCTGACAACTGTGCTCCTAGATGAGAACGGAGTGGGGC-3′; MUT3 (3305 to 3270), 5′-ACACCCTGACAACTGTGCTACTCGACGAAAATGGAG-3′; MUT4 (3305 to 3264), 5′-ACACCCTGACAACTGTGCTCCTAGCTGAAAATGGAGTGGGGC-3′; and No-MUT (3305 to 3270), 5′-ACACCCTGACAACTGTGCTCCTAGATGAAAATGGAG-3′; (iii) amplification of fragment *y*, PB 4U (3289 to 3308), 5′-CACAGTTGTCAGGGTGTTCG-3′; and PB 46 (4649 to 4630), 5′-GATACCATGGCGGGATCCAG-3′; (iv) amplification of fragment *z*, Ins (−669 to −650), 5′-TTCCTAG*AGATCT*GTACTGG-3′; and *Eco*RV (4120 to 4101), 5′-CTGTACGGTG*ATATC*ACACC-3′; (v) construction of deletion mutants, ΔAma 1 (5042 to 5057), 5′-CGCG*ACGCGT*AGGAGGGCCTCCAACAC-3′; ΔAma 2 (5033 to 5019), 5′-CGCG*ACGCGT*GCTGAAGCTGATCAAG-3′; ΔHir1 (5006 to 5022), 5′-CGCG*ACGCGT*TAGCCGCACTGACTATTC-3′; ΔHir2 (4997 to 4981), 5′-CGCG*ACGCGT*TAGCCGCACTGACTATTC-3′; Kp1 (4686 to 4705), 5′-TTGTGTA*GGTACC*GCTGTAT-3′; and Kp2 (2188 to 2169), 5′-AGACACT*GGTACC*AAGCGAC-3′; (vi) construction of fragments for EMSA, PBX (5073 to 5057), 5′-CGGGAGGAAAATTACTG-3′; PB1 (5055 to 5039), 5′-GTTGAGGCCCTCCGCC-3′; ERL4 (4934 to 4949), 5′-AGGGGATCCCGTAAGGGTAGAC-3′; PBIV-1 (3321 to 3300), 5′-GTCCTGCAGTTCACAAACACCC-3′; PB7-2 (3215 to 3235), 5′-GGGCGTCGACCCATTATATCTACACAGGAG-3′; YY1a, 5′-TCGGGAGGGTCTCCATTTTGAAGCGGGC-3′; and YY1b, 5′-CCGAGCCCGCTTCAAAATGGAGACCCTC-3′; (vii) DNA bending, Bend I (3265 to 3293), 5′-CTAGACCCCACTCCATTTTCATCTAGGAGCACAG-3′; and Bend II (3294 to 3264), 5′-TCGACTGTGCTCCTAGATGAAAATGGAGTGGGGT-3′; (viii) sequencing, PB549 (3478 to 3459), 5′-GGACATGGTCAACAAAGACC-3′; (ix) construction of pPyOriCMV YY1, DG1 (5157 to 5174), 5′-ACG*TCTAGA*CGCGTAGTTGCTAGGCAACTGG-3′; and DG2 (83 to 67), 5′-ACG*TCTAGA*GTCCTGTTTTGACAAG-3′.

Rate of recombination. Autoradiograms of blots were scanned with a densitometer (Corning 750 system) and peaks corresponding to the substrate (*S*) and product (*P*) were cut out and weighed on a precision balance. The rate of recombination was defined as the $P/(S + P)$ ratio, expressed as a percentage.

Virus growth curve. Virus arising from 3T6 cells transfected in 10-cm-diameter petri dishes with wild-type or mutant RmI was extracted after 12 days of incubation at 33°C using receptor-destroying enzyme (RDE) and assayed for hemagglutinating activity with guinea pig erythrocytes (10). Virus being virtually undetectable at this stage (<10 hemagglutinating units [HAU] per ml), the whole RDE extracts were used to infect 10-cm-diameter petri dishes of subconfluent 3T6 cells. After 8 days of incubation at 33°C, virus was again harvested and titrated. Titers in the range of 1×10^4 to 3.6×10^4 HAU per ml were observed, except for mutants MUT4RmI and MUT5RmI for which HAU were again undetectable. Growth curve analysis was then carried out by inoculating 5-cm-diameter petri dishes containing subconfluent 3T6 cells with 2 HAU of virus suspension, except for virus originating from MUT4RmI and MUT5RmI (<0.02 HAU per petri dish). Petri dishes were analyzed for virus content at 3, 6, 9, and 12 days postinfection, the RDE extracts being again titrated by hemagglutination.

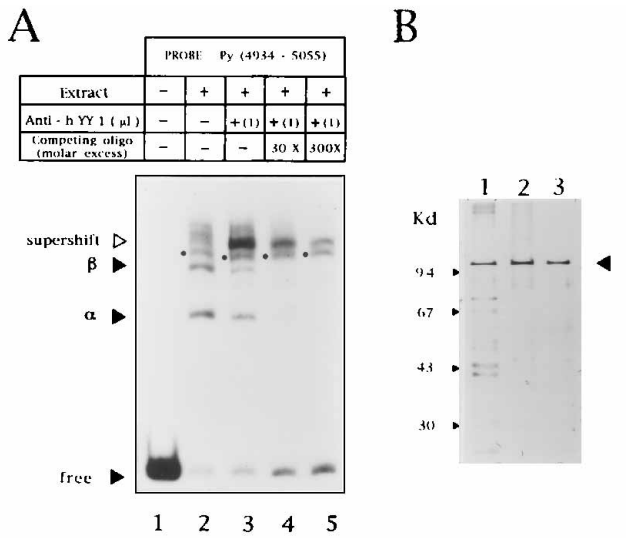

FIG. 3. Significance of gel retardation of DNA containing Y1 and Y2. (A) EMSA. The conditions selected were similar to those in Fig. 2C, especially with regard to lanes 1 to 3 in both figures. In Fig. 3A, however, conditions in lanes 4 and 5 were the same as in lane 3, except that a 30- or 300-fold molar excess of a double-stranded oligonucleotide (competing oligo) (YY1a plus YY1b; see Materials and Methods) including a strong YY1 binding site (52) was added to the radioactive probe carrying Y1 and Y2. Note that a spurious band (see black dots), presumably due to aging of the extract, is of constant intensity, under all reaction conditions. Note also that the amounts of free probe in lanes 4 and 5 suggest that the unlabelled oligonucleotide inhibits the formation of not only complexes α and β but also the species representing the supershift. (B) SDS-PAGE of proteins purified from *E. coli* expressing GST-YY1. In lane 1, protein had been purified by immunoprecipitation of 0.1 ml of bacterial lysate supernatant with 5 μl of anti-hYY1 antibody. Lanes 2 and 3 were run with 5 μl (or from 250 to 500 ng of protein) of two different preparations of GST-YY1 purified by chromatography on a glutathione-Sepharose column. The arrowhead on the right points to the band common to the three protein samples. The positions of molecular mass markers (in kilodaltons) are shown to the left of the gel.

Virus neutralization. In some experiments (see Fig. 7A), mouse anti-Py antiserum (M.A. Bioproducts) was added to the culture medium immediately after transfection. The concentration of antiserum in the culture medium (1:1,000) would have been sufficient to effect complete virus neutralization, assuming that 100 HAU or about 10^9 physical particles of virus would have been released in the medium of each 10-cm-diameter petri dish. Yet, HAU are generally undetectable in cultures having undergone transfection with RmI (see "Virus growth curve" above).

RESULTS

Protein binding to Y1 and Y2. A portion of Py DNA surrounding the unique *Bcl*I site at bp 5021 (Fig. 2A) was amplified by PCR with full-length RmI or RmI from which Y1 (9, 20) or Y2 (20) had been deleted (Materials and Methods) as the template. The PCR-synthesized DNA was then incubated with a protein extract prepared from mouse 3T6 cells and subjected to EMSA (Materials and Methods). The formation of a complex, designated α, was observed with all three DNAs, while an additional complex, designated β, was observed only with the full-length DNA (Fig. 2B). To further explore the significance of these findings, the two DNAs with different deletions, Δ site 1 and Δ site 2, were mixed prior to adding the protein extract and performing EMSA: only the α complex was observed under these conditions (not shown). We concluded from this observation that both Y1 and Y2 had to be present on the same DNA fragment for the β complex to be formed. Possibly, complex β migrated more slowly than complex α during electrophoresis because of the larger mass of the former.

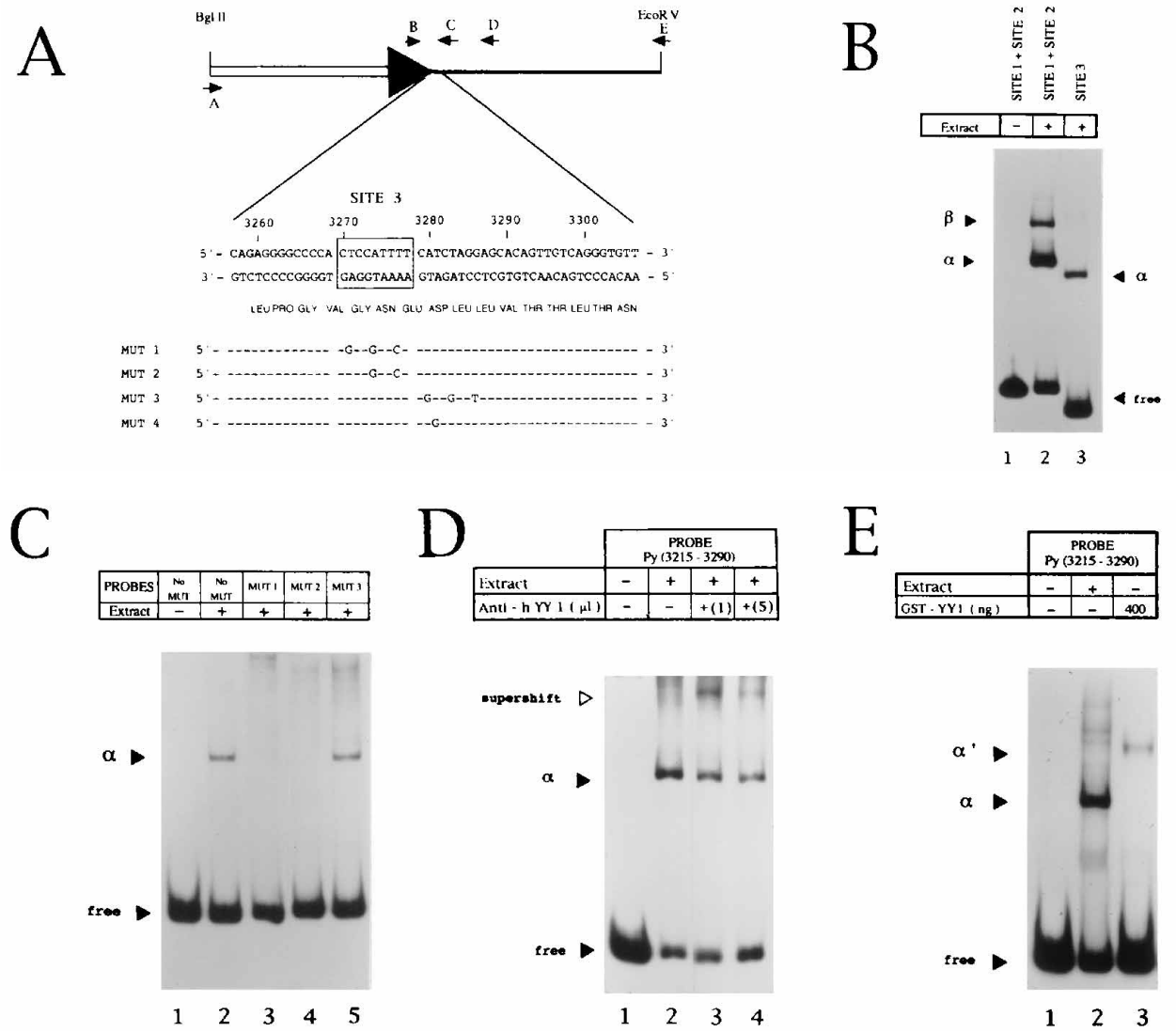

FIG. 4. A third YY1 binding site in Py DNA? (A) The map at the top shows the portion of RmI extending from the *Bgl*II site at bp −658 to the *Eco*RV site at bp 4106, which includes part of Ins (white box), repeat S_2 (large black arrowhead), as well as unique viral DNA (thick line) (Fig. 1). Short arrows show primers used for mutant construction (A or Ins, E or *Eco*RV), sequencing (D or PB549), or synthesis of EMSA probes (B or PB7-2, C or PBIV-1 [see Materials and Methods]). For the portion of the *Bgl*II-*Eco*RV fragment immediately surrounding Y3 (site 3), the figure shows from top to bottom, the double-stranded Py DNA sequence (15, 61), with the YY1 core sequence boxed, the corresponding amino acid sequence in VP1 (61), and finally substituted residues in the top strand of Py DNA (61) for four mutant RmI DNAs. Note that with the exception of MUT4, which converts Asp-266 in VP1 into an Ala (19; see also "Infectivity of RmI" and Fig. 8B), all mutations shown alter the third residue of each codon, leaving the amino acid specified unaltered. (B) EMSA carried out with DNA containing either Y1 and Y2 (primers A-B [Fig. 2A]) or Y3 (primers B-C [panel A]). Note that with Y3 (lane 3), only an α complex is formed. (C) EMSA with DNA synthesized from mutant RmI DNAs using primers B-C (panel A). Note that DNA synthesized from either MUT1RmI (lane 3) or MUT2RmI (lane 4), both of which carry mutations in Y3, does not give rise to an α complex, whereas DNA from MUT3RmI (lane 5), which carries three single-point mutations immediately adjacent to the YY1 site (see panel A), does give rise to such a complex. NoMUTRmI, which serves as a control (lanes 1 and 2), is a molecule constructed as MUT1, MUT2, or MUT3RmI, but using a nonmutant primer (see Materials and Methods). (D) Supershift. DNA amplified (primers B-C) from RmI was incubated with the extract from 3T6 cells in the absence (lane 2) or presence of 1 μl (lane 3) or 5 μl (lane 4) of anti-hYY1 antibody prior to EMSA. Note that the supershift is somewhat more evident with 1 than with 5 μl of anti-hYY1, as already noted with Y1/Y2 (Fig. 2C, lanes 3 and 4). (E) EMSA with crude mouse nuclear extract or GST-YY1. Note the α′ complex formed with GST-YY1 (lane 3).

Alternatively, β-complex formation could depend upon Y1 and Y2 interacting with one another through bound YY1, while the intervening DNA was looping out. The subsequent observation that efficient β-complex formation appeared to require a minimal distance between Y1 and Y2 does not allow us to decide which of the two interpretations was more likely to account for our result (15).

Next, we performed EMSA with only the full-length DNA fragment, but in the presence of both the protein extract from 3T6 cells and antibody directed against human YY1 (anti-hYY1) (Fig. 2C). A supershift was then clearly observed, indicating recognition by the antibody of a DNA-bound mouse protein immunologically related to YY1, presumably δ (18). In contrast, no supershift was observed with KT3 (30), a monoclonal antibody directed against an unrelated protein, simian virus 40 (SV40) large T antigen (Fig. 2C). Finally, EMSA was performed with purified GST-YY1 instead of the crude 3T6 nuclear extract. As expected from the higher molecular weight of GST-YY1, two larger complexes, designated α′ and β′ here, were formed with DNA carrying both Y1 and Y2 (Fig. 2D).

In Fig. 3, we present two additional pieces of data supporting the notion that both complexes noted with either crude mouse nuclear extract (α and β) or GST-YY1 (α′ and β′) reflect the interaction of the DNA probe with a protein with the binding specificity of YY1 or δ. First, in the presence of a constant amount of anti-hYY1 antibody, increasing amounts of an oligonucleotide carrying a strong YY1 binding site, inhibited the formation of not only complex α and complex β but also the band resulting from the supershift (Fig. 3A). The fact that, in particular, adding increasing amounts of the oligonucleotide to the reaction mixture resulted in the progressive fading of the band representing the supershift clearly suggested that the antibody used acted through its anti-YY1 or anti-δ specificity (Fig. 3A). Also, the degree of purity of our GST-YY1 preparations (Fig. 3B) represented further assurance that the α′ and β′ complexes (Fig. 2D) owed their electrophoretic properties to GST-YY1 rather than to some contaminant *E. coli* protein. Therefore, together with those obtained with Δ site 1 and Δ site 2 (Fig. 2), these data clearly indicated that Y1 and Y2 were indeed true YY1 binding sites.

A third YY1 binding site. Scanning of the sequence of Py DNA (or RmI) indicated the presence of a possible YY1 binding site spanning viral bp 3270 to 3278, and thus overlapping with S_2 in RmI (Fig. 1 and 4A). We investigated how DNA including this site would behave in EMSA compared with DNA carrying Y1 or Y2. Thus, DNA including viral bp 3270 to 3278 was found to give rise to an α complex but not a β complex (Fig. 4B). To confirm that we were indeed dealing with a YY1 binding site and also to produce DNA to allow us to assess whether our finding had any biological significance, we generated point mutations in the portion of RmI centered on bp 3270 to 3278 (see Materials and Methods). It is important to realize that the sequence from bp 3270 to 3278, which partly overlaps with that of S_2, is present once in either Py DNA or RmI (Fig. 1 and 4A). Probes synthesized from three mutant RmI DNAs and used in EMSA demonstrated that either of two mutations (MUT1 or MUT2) within the presumptive YY1 binding site designated Y3 abolished complex formation, while DNA carrying three single-base substitutions in the immediate vicinity of Y3 (MUT3), or control DNA generated by the same strategy as the mutants but carrying no mutation at or near Y3 (NoMUT), band shifted normally (Fig. 4C). In order to assess the significance of these data, we investigated whether the complex including Y3 could be supershifted with anti-hYY1 antibodies. As already observed with the DNA probe including Y1 and Y2, a supershift was observed with the Y3-containing probe, with the corresponding band stronger with 1 than 5 μl of anti-hYY1 (Fig. 4D and 2C). Furthermore, using the Y3-containing probe with GST-YY1 instead of a crude nuclear extract in EMSA converted the single α band into what appeared to be an α′ band (compare Fig. 4E with 2D). Also, we carried out experiments in which we compared the ability of various DNAs or oligonucleotides to inhibit the protein(s) shifting the Y3 probe during EMSA. The results obtained (Fig. 5) suggested that the protein from our extracts which bound to Y3 had the same binding specificity as that binding to the YY1 binding site in the P5+1 element of the P5 promoter from adeno-associated virus type 2 (52). Finally, since YY1 has been shown to bend DNA in vitro (37), we tested whether DNA including Y3 would bend (26) in the presence of our mouse nuclear extract. We found that indeed such DNA was clearly bent in the presence of the protein extract and not in its absence (Fig. 6A and B). Significantly, the center of bending appeared to be located within the Y3 sequence itself (Fig. 6C and D).

Release of Py DNA from mutant RmI. RmI and mutants of RmI characterized above were excised from the corresponding

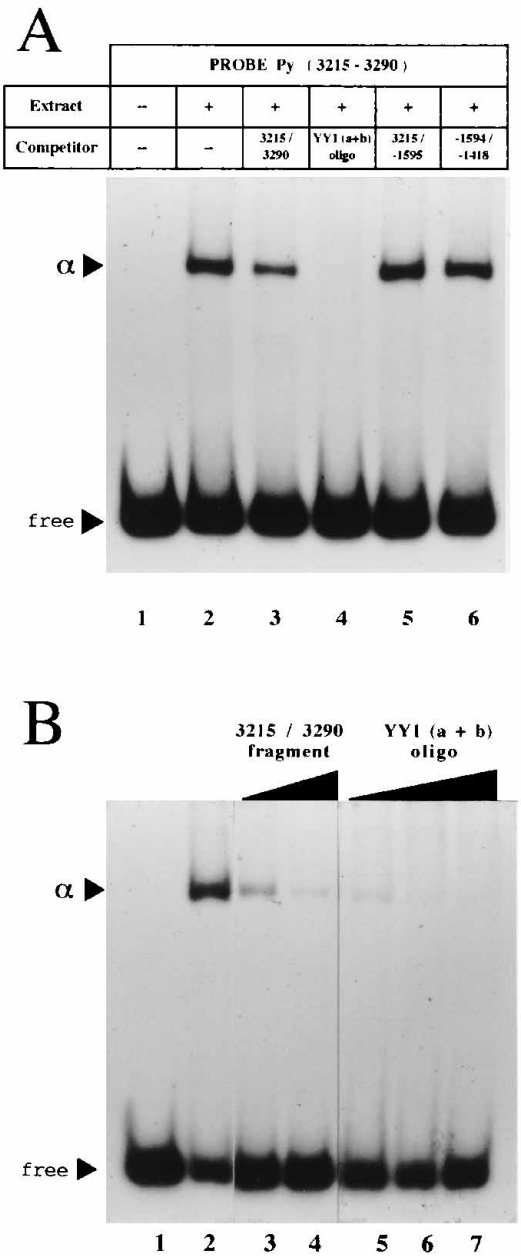

FIG. 5. Competition of various DNA fragments or oligonucleotides with Y3. The radioactive Y3 probe used in Fig. 4 was mixed with various competitors and the mouse protein extract prior to EMSA. (A) Radioactive Y3 against a set amount (100-fold molar excess) of cold competitor. While two controls were run in lanes 1 and 2, the cold competitor in lane 3 was the same DNA as that used to make the probe. Other DNA competitors contained either the juncture between repeat S_1 and Ins (lane 5) or part of Ins (lane 6) (Fig. 1A). Only in lane 4 does the competitor consist of two annealed oligonucleotides (YY1a and YY1b [Fig. 3A]) instead of PCR-synthesized DNA. Note the particularly strong competition between Y3 and such a prototype YY1 element (52). (B) Competition between radioactive Y3 DNA and cold Y3 DNA or the YY1a plus YY1b double-stranded oligonucleotide (oligo). Lanes 1 and 2 are similar to the same two lanes in panel A, while mixtures containing competitor DNA or oligonucleotide were run in the other lanes. Molar excesses were 100- and 300-fold (lanes 3 and 4, respectively) and 30-, 100- and 300-fold (lanes 5, 6, and 7, respectively). More vigorous competition by the YY1a plus YY1b oligonucleotide than by the cold PCR fragment (bp 3215 to 3290) may be due to the shortness or greater purity of the oligonucleotide; alternatively, the prototype YY1 binding site (52) on which the oligonucleotide was tailored (Materials and Methods) could be particularly strong.

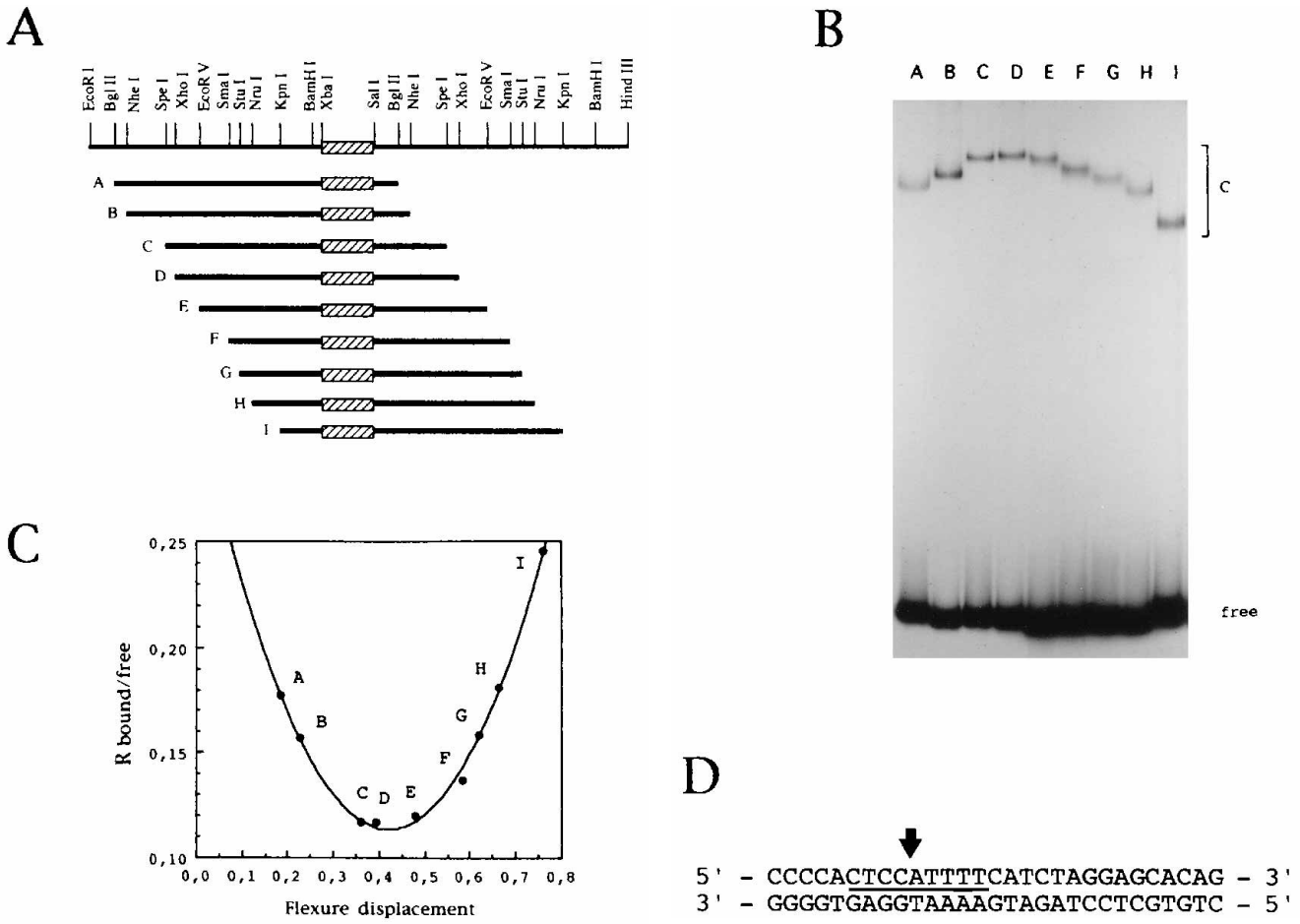

FIG. 6. DNA bending at Y3. (A) Schematic representations of circularly permuted DNA fragments used in the assay. The top line represents part of vector pBend2 (26) with the oligonucleotide containing Y3 (hatched box) cloned between sites *Xba*I and *Sal*I (see "DNA bending" above). Digestion with the following restriction enzymes generated circularly permuted fragments, each 150 bp long: *Bgl*II (A), *Nhe*I (B), *Spe*I (C), *Xho*I (D), *Eco*RV (E), *Sma*I (F), *Stu*I (G), *Nru*I (H), and *Kpn*I (I). (B) Electrophoretic analysis of circularly permuted protein-DNA complexes. ^{32}P-labelled DNA fragments (A to I) were mixed with a nuclear extract from mouse 3T6 cells and subjected to EMSA as detailed in Materials and Methods. C, complexed DNA. (C) Mapping the bending site within the oligonucleotide inserted into pBend2. Mobilities of protein-DNA complexes were normalized to the mobility of the corresponding free DNA ($R_{bound/free}$) and plotted against the flexure displacement (distance of the center of Y3 from the 5′ end of the probe divided by the total length of the probe). The bend center was estimated by extrapolating the linear portions of the curve to a virtual point on the horizontal axis. (D) Nucleotide sequence of the oligonucleotide inserted into pBend2. Y3 is underlined. The vertical arrow marks the center of bending.

recombinant plasmids via *Sal*I digestion. The various inserts were then circularized with ligase and transfected into permissive mouse 3T6 cells. Following 3 or 6 days of incubation at 33°C, low-molecular-weight DNA was extracted from the monolayers by the method of Hirt (21) and analyzed by the Southern (55) procedure (Fig. 7). Relating the amount of product to the total amount of viral DNA suggested for the first experiment (Fig. 7A) that MUT1RmI, which carries three single-base substitutions in Y3 (Fig. 4A), is converted into 5.3-kb Py DNA less readily than MUT3RmI, which carries a 3-bp substitution outside Y3 (Fig. 4A), or NoMUTRmI, a nonmutated RmI obtained by the same procedure as the mutated RmI DNAs (see Materials and Methods and "A third YY1 binding site" above). Actually, comparing MUT1RmI with MUT3RmI indicated that the conversion of substrate into product was three to four times less effective for the former than for the latter of these two substrates (Fig. 7A). In a subsequent experiment, conversion of substrate into product was determined for not only MUT1, MUT3, and NoMUTRmI but also MUT2RmI, which carries two point mutations in Y3 (Fig. 4A). This time, product release was decreased 3- to 5-fold for MUT2 and 7- to 10-fold for MUT1RmI relative to that of either MUT3 or NoMUTRmI (Fig. 7B and C). Thus, mutations in Y3 appeared to consistently decrease the release of Py DNA from RmI. It is important to realize at this stage that the mutant RmI DNAs described thus far had been constructed in the hope of detecting alterations in a presumably *cis*-acting process. Indeed, none of the mutations deliberately introduced into RmI DNA could have affected the amino acid sequence of the only protein known to be encoded by the given region, VP1 (Fig. 4A).

Infectivity of RmI. Reduced production of Py DNA from RmI with a mutated Y3 site was consistent with this site being required for stimulation of recombination. Other less likely explanations, however, were not formally excluded. For instance, the inability of YY1 to bind to Y3 could disturb transcription elongation, thus causing premature termination of late transcripts and, indirectly, depression of VP1 synthesis by Py genomes. Ineffective packaging of Py DNA would then ensue and restrict subsequent virus propagation, such that a lower virus yield would mimic a reduced recombination rate. Alternatively, mutagenesis by PCR might have produced not

only the desired mutations but also some unwanted ones lying outside the region subjected to sequencing. Such additional mutations could have inactivated either VP1, VP2, or VP3 (61), again restricting virus propagation. Since the only real choice was between an effect on recombination and an effect on virus propagation, we decided to investigate such propagation after transfection with mutated or nonmutated RmI. In the first experiment (Fig. 8A), mouse 3T6 cells were transfected with either RmI, MUT1RmI, or MUT3RmI and incubated in our regular tissue culture medium (−AS) or medium supplemented with antibodies directed against Py (+AS; see Materials and Methods). Six days later, DNA was extracted from the cells and analyzed by the Southern procedure (55) as described in the legend to Fig. 7A and B. Thus, we were unable to detect in the −AS and +AS medium any differences in the relative amounts of Py DNA generated large enough to suggest that a substantial fraction of that DNA was attributable to virus propagation rather than recombination. Indeed, if such propagation had taken place under our conditions, it would have affected the relative amounts of Py DNA detected not only for the mutant but also for the nonmutant recombination templates. However, the differences previously noted between the different templates in terms of yield of recombination product—or Py DNA—remained the same (Fig. 8A) as noted before (Fig. 7). This result already suggested that viral genomes carrying silent mutations in the VP1-coding gene spread through the cultures as readily as viral genomes carrying no such mutations. This conclusion was confirmed in the second experiment, in which a set amount of virus originating from RmI, either wild type or mutant, was inoculated into 3T6 cultures. These cultures were harvested after 3, 6, 9, or 12 days of incubation at 33°C and digested with RDE, and the resulting yield of Py was then assessed by hemagglutination (10). Such an experiment indicated that all viruses originating from RmI-like molecules which were expected to include no change in protein coding capacity were indeed propagated at similar rates. In contrast, for RmI-like molecules that could give rise only to Py DNA encoding a mutant VP1 polypeptide, such as MUT4RmI and MUT5RmI (Fig. 4A and Materials and Methods), no virus growth could be observed (Fig. 8B). Altogether, the data displayed in Fig. 8 demonstrated that whether originating from RmI, NoMUTRmI, or any mutant RmI with an intact protein coding capacity, Py DNA—or Py—would replicate at approximately the same rate. Therefore, the rate of generation of Py DNA could vary only between these various templates with the rate of recombination characteristic of each.

A specific role for Y3 in recombination. The data presented in Fig. 7 and 8 could be readily reconciled with only one interpretation, i.e., mutations in Y3 exert a *cis*-acting inhibitory effect on recombination in RmI. Indeed, rapid reversion of these mutations was unlikely, in view of the total inactivation of the templates carrying mutations close to Y3 such as MUT4 and MUT5 (Fig. 8B), particularly MUT4, which carried alteration of 1 bp, resulting in a single-amino-acid substitution (Fig. 4A). However, our data thus far did not allow us to decide between a general effect of Y3 on recombination, due for instance to an overall change in conformation of the recombination template, and a more specific, perhaps local, effect. In a previous report (38), we had demonstrated the usefulness of constructs derived from RmI but containing two viral regulatory regions instead of one (Fig. 9A). Such constructs, termed O/RmI, can undergo one of two mutually exclusive recombination events, LR, involving the large segments surrounding the two viral regulatory regions, and SR, involving the two S repeats (Fig. 9B). Since each of the possible products would necessarily include a functional Py origin (Fig. 9B), SR and LR taking place simultaneously in a transfected monolayer would necessarily generate four new amplifiable molecular species (T, B, L, and R [Fig. 9B]), all readily separable from the O/RmI precursor. In Fig. 9C, we demonstrate that O/RmI carrying an intact Y3 indeed yields four recombination products (T, 5.3 kb; B, 3.7 kb; L, 4.9 kb; and R, 4.1 kb) following transfection of mouse 3T6 cells. Note that whether SR or LR is involved, the two reciprocal products are registered in about equal amounts (Fig. 9C). This type of pattern has already been described before (38). Strikingly however, with O/RmI carrying

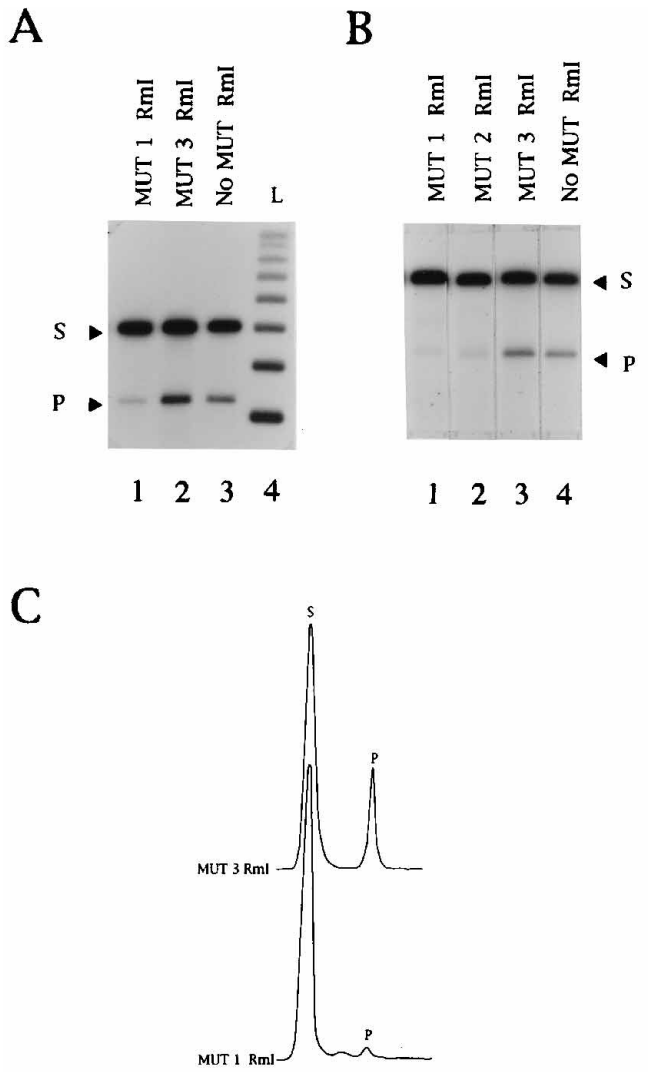

FIG. 7. Effects of point mutations on recombination of RmI. (A) Transfection of 3T6 cells with MUT1 (lane 1), MUT3 (lane 2), and NoMUTRmI (lane 3). After incubation for 6 days at 33°C, DNA was extracted by the method of Hirt (21), deproteinized, and digested with *Dpn*I and *Eco*RI before being electrophoresed, blotted, and hybridized with a radioactive viral probe, as described in Materials and Methods. S, recombination substrate (RmI); P, recombination product (Py DNA). Lane 4 was run with a radioactive molecular size marker (ladder [L]). (B) Results from another experiment carried out with the same mutants as in panel A, plus MUT2RmI (lane 2). (C) Scanning of lanes 1 (MUT1RmI) and 3 (MUT3RmI) of the autoradiogram shown in panel B. The four $P/(S + P)$ (P is product and S is substrate) ratios were as follows: MUT1RmI, 2.3%; MUT2RmI, 4.8%; MUT3RmI, 22.8%; and NoMUTRmI, 16.4% (the difference between the ratios for MUT3RmI and NoMUTRmI is believed not to be significant). Mutations MUT1, MUT2, and MUT3 are shown in Fig. 4A.

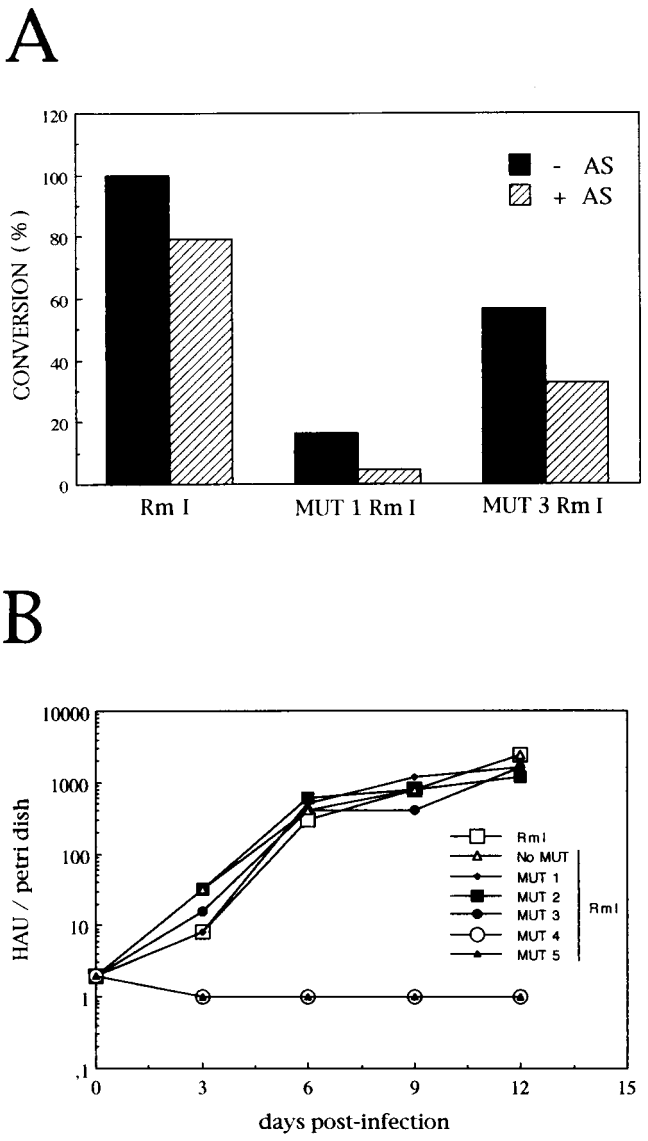

FIG. 8. Differences between RmI and its mutants are not due to DNA alterations curtailing virus propagation. (A) Generation of Py DNA from RmI in the absence or presence of anti-Py antiserum. After monolayers of 3T6 cells had been transfected with either RmI, MUT1RmI, or MUT3RmI and incubated at 33°C for 6 days in medium with (+AS) or without (−AS) anti-Py antiserum, low-molecular-weight DNA was selectively extracted, digested with *Dpn*I and *Eco*RI, subjected to electrophoresis, blotted, and annealed with a viral DNA probe. The autoradiogram was then scanned with a densitometer (Materials and Methods). The histogram shows the extent of conversion of the template into Py DNA [$P/(P + S)$ ratio (Fig. 7)] for the various RmI DNAs. The conversion rates are expressed as percentages of the highest value registered (RmI, −AS). (B) Growth curves. Aliquots (2 HAU) of virus originating from cells transfected with RmI or one of its derivatives (MUT1, MUT2, MUT3, or NoMUTRmI) were inoculated into 5-cm-diameter petri dishes containing monolayers of mouse 3T6 cells. After 3, 6, 9, or 12 days of incubation at 33°C in Dulbecco modified Eagle medium containing 2% fetal calf serum, pairs of petri dishes were analyzed for virus content. Total yields of HAU per petri dish are shown. For controls, parallel cultures were inoculated with blind but parallel passages from cells initially transfected with MUT4RmI and MUT5RmI, in which no HAU could be measured. HAU remained undetectable in these cultures. MUT4 (Fig. 4A), which substitutes an Ala for an Asp in VP1, has already been shown to prevent capsid assembly (19). MUT5RmI combines two mutations, MUT1 and MUT4 (see Materials and Methods and Fig. 4A).

the MUT1 mutation, only three products were observed: L, R, and B. The fact that L and R were released from all constructs tested (Fig. 9C) indicates that none of the mutations generated in this work had any impact on LR, that is, recombination between the two viral regulatory regions, both of which were remote from Y3 (Fig. 9A). This observation forms a striking contrast with the effect of mutating the promoters in O/RmI, which can have a marked effect on LR (38). Here, the fact that O/MUT1RmI was the only one of the four templates tested for both SR and LR to generate the B product but not the T product (Fig. 9C, lane 3) suggested that Y3 acts specifically on SR. However, we found it remarkable that an intact Y3 was required only for the generation of the T product, in which it is eventually included (Fig. 9B). Finally, the failure of O/MUT1RmI to generate the T product could not be attributed to the inability of this product to replicate, since all O/RmI molecules had been made by introduction of the same fragment of DNA into the corresponding RmI (see Materials and Methods and Fig. 9). Release of B without T, while being consistent with previous results already indicating that SR is a nonconservative process (38), also suggests that recombination between S repeats is driven—or enhanced—by elements lying outside the repeats themselves. It was then even more remarkable that these elements, as well as some others distant from the S repeats (see Discussion), were all located within the DNA to be included in the amplifiable recombination product that happened to be formed.

Cotransfection of RmI or O/RmI with vectors allowing overexpression of YY1. In an attempt to ascertain whether the interaction of YY1 with Y3 was instrumental in promoting the generation of Py DNA from RmI or O/RmI, each of the latter two molecules was cotransfected into mouse 3T6 cells with either one of two vectors likely to express the YY1 gene under the control of a strong promoter; in each case, vectors carrying the YY1 gene in the minus-sense orientation, which is not supposed to allow gene expression, were also transfected with the recombination template (see Materials and Methods). Also, both vectors included a Py DNA regulatory region (see Materials and Methods), so that they would replicate (and presumably overexpress YY1) but only in cells (mouse 3T6) in which the simultaneous presence of the recorder plasmid (RmI or O/RmI) would allow the replication of the expression vector. We noted that indeed, replication of O/RmI or RmI was accompanied by that of the YY1-expressing vector after cotransfection of mouse 3T6 cells (15). Northern (RNA) blots also indicated that the YY1 message was present in increased amounts in cells transfected with vectors carrying the YY1 gene in the plus-sense but not minus-sense orientation (15). However, the presence and amplification of the YY1-expressing vector (plus-sense orientation) had no detectable effect on the replication or recombination of either RmI or O/RmI (15). Such ambiguous results are not really surprising and surely not inconsistent with YY1 being the protein whose binding to Y3 is needed for the release of Py DNA from RmI or O/RmI (see Discussion).

DISCUSSION

YY1 binding sites in Py DNA. Data included in this report support and extend results from two other laboratories (9, 20) which indicated that the region containing the enhancer and late promoter of Py DNA carries two YY1 binding sites flanking the unique *Bcl*I restriction site at bp 5021. This finding is not particularly surprising, in view of the fact that while the region containing the enhancer and late promoter of Py DNA is often shown as if extending from the origin to the unique

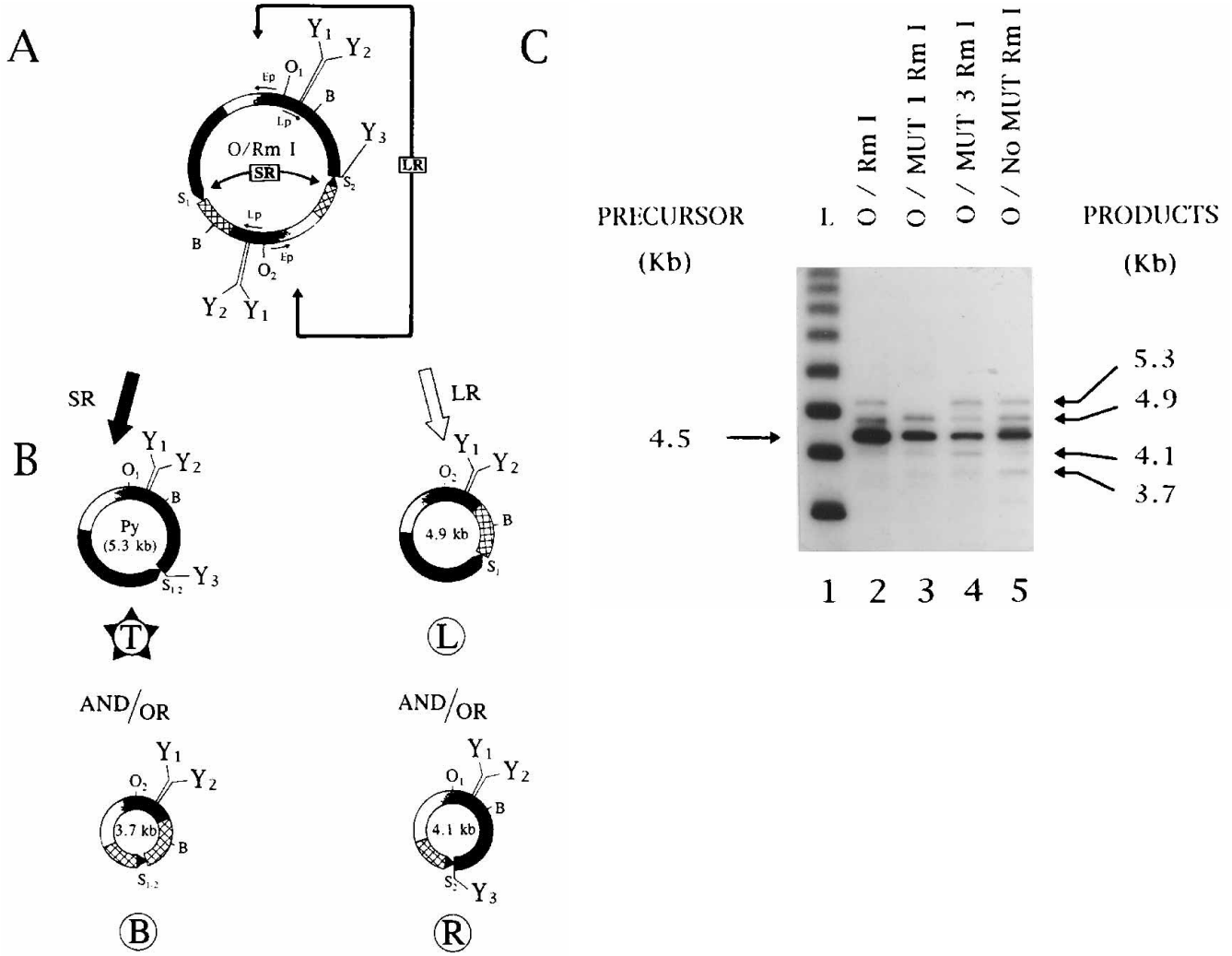

FIG. 9. Recombination pattern of O/RmI and its mutants. (A) Map of O/RmI. O/RmI is basically RmI (Fig. 1) carrying, at the *Bgl*II site of Ins, a Py DNA fragment encompassing the origin (O_2) in the same orientation as the identical fragment of RmI (see O_1 and orientation of shaded boxes). O/RmI can undergo one of two homologous recombination events, one involving S_1 and S_2 (SR) and the other involving the large direct repeats centered on O_1 and O_2 (LR). (B) Products expected from SR or LR. Note that each of the four (T, B, L, and R) possible products includes a complete viral intergenic region, making it readily amplifiable, as well as one of the two *Bam*HI (B) sites existing in O/RmI. Note that Y3 is expected to be part of T and/or R, but that only in T (indicated by a 5-point star) would it be positioned next to a recombination juncture (S_1-S_2). (C) Recombination products of O/RmI and its mutants. O/RmI and three of its mutants were transfected into *Scop*-T1 cells (43), and the recombination patterns were analyzed as described in the legend to Fig. 7A, except that the Hirt-extracted DNAs were digested with *Bam*HI instead of *Eco*RI. Note that all constructs give rise to a 3.7-kb product, while a 5.3-kb or T product (see panel B) is noted for all constructs, except O/MUT1RmI (the only construct to include a mutated Y3 site [Fig. 4A]). The strong 4.5-kb band originates from the twice-cut (see panel A) precursor. Lane 1 was run with a molecular size marker (ladder [L]).

*Bcl*I site (35, 63, 67), several results suggest the existence of transcriptional regulatory elements further downstream of the origin (4, 35). Y1 and Y2 could represent *cis*-regulatory sequences belonging to either the enhancer or late promoter, depending on whether enhancer function is indeed dispensable for late promoter activity (4) and whether the portion of Py DNA spanning bp 4632 to 5021 upregulates the activity of the early promoter (20). The distance between Y1 and Y2 is neither very different from that observed between YY1 sites in other promoters (53) nor too large for these sites to represent enhansons within the same enhancer (13). Sites Y1 and Y2 are thus likely to function coordinately in the regulation of transcription, while some of our data suggest that they may play an as yet unspecified role in recombination (15). In view of the complexity of the regulatory role played by the region of Py DNA surrounding the *Bcl*I site (4, 9, 23, 35, 61, 63, 67), however, we prefer, at this stage of our work, to postpone any discussion regarding the possible involvement of Y1 and Y2 in recombination. Y3 poses an entirely different problem. Indeed, Y3 is located about 1.7 kb downstream of Y2, well within the late coding region, and one may wonder if it is involved at all in the regulation of transcription initiation as already noted previously for other intragenic YY1 sites (2, 18, 66).

Role of Y3 in recombination. Our data clearly suggest that point mutations interfering with the binding of YY1 to Y3 in vitro strongly depress the in vivo generation of Py DNA from RmI. Of course, the observed ratio between RmI and Py DNA depends on both the rate of recombination of RmI and the rate of replication of each of these two molecules. However, the mutations introduced into Y3, with the possible exception of the transversion at bp 3271 in MUT1 (Fig. 4A), should be faithfully transferred from RmI into Py DNA (Fig. 1). Thus, an alteration affecting replication would presumably depress replication of both the template and the product, so that the ratio

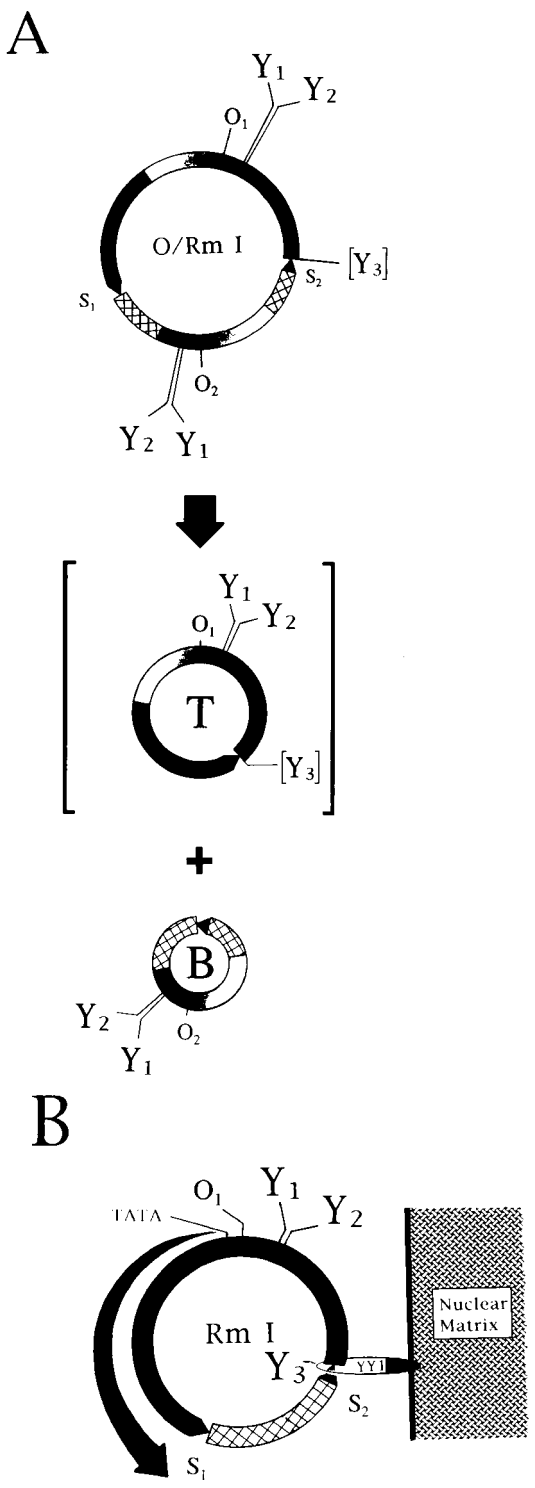

FIG. 10. Role of Y3 in recombination. (A) Schematic representation of SR in O/RmI. Viral sequences are shown as arrowheads (S_1 and S_2) or shaded boxes and mouse sequences are shown as checkered boxes, as done in Fig. 9. While O/RmI with an intact Y3 site yields two reciprocal products, both amplifiable, T and B, O/RmI with a mutated Y3 site [Y_3] yields a B product but not a T product (brackets). In contrast, LR remains unaltered even though Y3 is mutated (Fig. 9C). (B) Hypothetical model for the involvement of Y3 in recombination at the S_1/S_2 hot spot. The molecule shown is RmI and the basic symbols are the same as in panel A. Transcriptionally active RmI is tethered by YY1 (loop) to the nuclear matrix. In the case illustrated, Y3 is involved only in such tethering, and binding of YY1 to this site results not only in bending of Y3 but also in denaturation of S_2 (not shown; see Discussion). Transcription (large curved arrow) from the early promoter (TATA) either generates positive supercoils ahead of the transcription complex, favoring denaturation of S_1, or displaces the anti-early DNA strand within S_1 (38, 39). Either way, pairing and/or strand exchange is prompted between S_1 and S_2 (38, 39). This model is consistent with not only the phenotypes of Y3 mutants but also with our earlier observation that an intact early promoter is needed specifically for SR (7, 39).

between the two would tend to remain constant. Also, the virus growth curves which we established are essentially consistent with the assumption that the sequence alterations which we have introduced into the template for recombination have no significant effect on DNA replication or even virus replication (Fig. 8). Finally, experiments carried out with constructs of the O/RmI type confirmed that Y3 acts specifically on recombination and not on the ability of the recombination product(s) to replicate (Fig. 9). Indeed, mutations in Y3 reduced the occurrence of product T (Py DNA), but not that of product R (Fig. 9C), although these two products, while occurring via different pathways, both carried the same altered Y3 site (Fig. 9A and B). Even more remarkably, alterations in Y3 reduced the occurrence of product T, but not that of product B, one product being the reciprocal of the other (Fig. 9). Therefore, an intact Y3 site appears to be required for the formation of the S_1-S_2 juncture, but only with respect to the product of which the Y3 site is part. Such *cis* effect of Y3 on product release is consistent with the model which we propose to account for intramolecular recombination in RmI or O/RmI (38, 39; see below).

Possible role of YY1 in recombination. At the suggestion of reviewers of this article, we have attempted to ascertain whether overexpression of YY1 mRNA or anti-YY1 mRNA would have any effect on recombination in RmI or O/RmI. Because recombination of RmI or O/RmI becomes detectable only after 3 to 5 days following transfection, we included a Py DNA origin of replication in expression vectors which had already proven their effectiveness in various short time assays (see Materials and Methods). Even though cotransfection of the reporter construct (RmI or O/RmI) with such a modified expression vector(s) was followed by detectable expression of the sequence of interest, no effect on recombination of the reporter construct could be observed. There are several possible explanations for such a negative result. First, the protein overexpressed in mouse cells was human YY1 and thus not active in a foreign context. Yet, the human YY1 encoded by our vector had already been shown to be capable of regulating transcription in murine cells (41, 44, 49). Alternatively, YY1 expressed from the vector could be of little or no consequence to the overall concentration of YY1 in the cell or to that of the species of YY1 involved in recombination. This assumption is not unreasonable, in view of several pieces of data. First, YY1 overexpression could not be shown to consistently affect the activity of some promoters known to be YY1 dependent (32, 36), possibly because functionally saturating amounts of YY1 are present in a number of cell lines (36). Furthermore, the facts that YY1 seems able to regulate transcription even in the absence of readily detectable YY1 binding sites (18, 44), interacts with a number of regulatory proteins (27, 28, 50, 54), and assumes several different roles in transcription regulation (49, 51, 52) would be consistent with the existence within the mammalian cell of more than one species, form, or state of this protein. Finally, the nuclear matrix protein NMP-1 has recently been shown to be indistinguishable from YY1 (17). The nuclear matrix is known to play a role in defining the higher-order structure of chromatin and the tethering of actively transcribed genes via so-called matrix-associated regions or scaffold-attached regions in the DNA (reference 17 and references there-

in). Besides explaining at least in part the formation of complexes between YY1 and nuclear matrix-associated proteins (references 17 and 62 and references therein) such as c-Myc (54), E1A (28, 52), or Sp1 (27, 50), all these findings suggest the existence of at least two forms of YY1 with distinct biochemical properties and/or partition in distinct nuclear compartments (17, 62). Tethering of Y3 to the matrix via YY1 could have a number of consequences, such as local unwinding and bending of the DNA (see reference 11 and references therein), which in turn could favor recombination between the S repeats (34).

Model for recombination. The selective effect of mutations in Y3 on recombination can be readily reconciled with our model of transcription-dependent recombination of Py DNA (38, 39) (Fig. 10). Perhaps significantly, Y3 is conserved (15) in the DNAs of all isolates of Py which have been sequenced (47), while such conservation is not needed for viral DNA replication, VP1 function, or more generally virus propagation, as the results reported here do indicate. According to our data (Fig. 6), Y3 is not an intrinsically bent site on the DNA, so that loss of intrinsic bending can hardly be invoked to explain the effect of our mutations on recombination (34). The sequence of SV40 DNA does not suggest that this DNA carries any YY1 site (15). However, SV40 DNA contains several sites for transcription factor Sp1 (14), a protein binding to YY1 (27, 50) and also to the nuclear matrix (62). Thus, Sp1 could conceivably play for SV40 the role assumed by YY1 for Py. With both factors being relatively ubiquitous, the functions of factors such as YY1 or Sp1 could be dictated in part by other proteins or factors, some of which could be cell specific (49). While SV40 and Py are often described as viruses with different host specificities, our remark concerning Sp1 and YY1 sites might also indicate that they are viruses with different cell or organ specificities (45).

A last point to be made concerns the mechanism generating or eliminating sites for transcription factors like YY1 or Sp1. Enhancers generally contain multiple binding sites for such factors, with such multiplicity often resulting from duplication of discrete DNA segments (13). Actually, the Py DNA enhancer offers examples of additional binding sites which are created not within the duplicated segment itself but by joining this segment to the preexisting DNA (9). RmI, the source of all constructs studied here, seems to arise via the opposite sequence of events, since its excision from the host cell chromosome involves the disappearance rather than the appearance of a new YY1 site (58). Indeed, excising RmI involves joining to bp −1628 of Ins, bp 3273 of repeat S_1 (Fig. 1), and thus interrupting the Y3 site adjacent to repeat S_1 in the integrated viral structure (58) (Fig. 4A). Thus, binding of YY1 to Y3 might be required not only for the release of Py DNA from RmI but also for that of RmI from the transformed host cell chromosome (58).

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