The retroviral integrase catalyzes two successive chemical reactions essential for integration of the retroviral genome into a host chromosome: 3′ end processing, in which a dinucleotide is cleaved from each 3′ end of the viral DNA; and the integration reaction itself, in which the resulting recessed 3′ ends of the viral DNA are joined to the host DNA. We have examined the stereospecificity of human immunodeficiency virus type 1 integrase for phosphorothioate substrates in these reactions and in a third reaction, disintegration, which is macroscopically the reverse of integration. Integrase preferentially catalyzed end processing and integration of a substrate with the (R)-phosphorothioate stereoisomer at the reaction center and disintegration of a substrate with an (S)-phosphorothioate at the reaction center. These results suggest a model for the architecture of the active site of integrase, and its interactions with key features of the viral and target DNA.

Integration of the retroviral genome into a host cell chromosome is required for viral replication (1). To accomplish integration, integrase, a viral enzyme, catalyzes two chemical reactions. In the first reaction, end processing, integrase cleaves the terminal dinucleotide from each 3′ end of the double-stranded viral DNA (Fig. 1A). In the second chemical reaction, integration or DNA joining, integrase joins the recessed 3′ ends of the viral DNA to target DNA (Fig. 1B). Integration and short sequences at each end of the viral DNA are required for these two reactions. After the viral DNA 3′ ends are joined to host DNA, the 5′ ends of the viral DNA are trimmed and joined to host DNA by a mechanism that remains to be defined (3).

In vitro assays, using duplex oligonucleotides that mimic viral DNA ends and highly purified, recombinant integrase, have been developed to allow detailed analysis of the end-processing and integration reactions (4). Both the end-processing and integration reactions carried out by HIV-1 integrase produce (S)-phosphorothioate products from model substrates containing an (R)-phosphorothioate at the reaction center, implying that both reactions occur by a one-step transesterification mechanism (5).

The available evidence supports a model in which a single active site is responsible for both reactions (6–13). The orientation and alteration of the DNA substrates for these two different reactions thus presents an interesting puzzle, as elaborated below.

End processing and integration are both polynucleotidyl transfer reactions, but the identity and organization of the reactants relative to the attacking hydroxyl nucleophile and the leaving group are different. In end processing (Fig. 1A), a specific phosphodiester bond in the viral DNA is attacked by a hydroxyl derived from water (HOH) and the leaving group is the 3′ hydroxyl of the conserved, subterminal adenosine; in integration (Fig. 1B), this newly formed 3′ hydroxyl is the attacking nucleophile. Moreover, in end processing, the phosphate group undergoing substitution is initially part of the viral DNA end and is released as part of a dinucleotide product after cleavage, while in integration, the phosphate that is attacked is initially part of a separate target DNA substrate that then becomes covalently joined to the viral DNA end.

The differences between end processing and integration make it difficult to envision how the DNA substrates in the two reactions could retain a normal double-helical structure and still be accommodated by the same active site. Experimental evidence suggests that the terminal 3 nucleotides of model viral DNA substrates are indeed unpaired prior to end processing (14). Highly bent sites in DNA are preferred targets for integration, a finding consistent with a need for distortion of the DNA duplex to accommodate the reacting phosphate group in the active site (15–19).

Integrase can catalyze a third reaction in vitro, termed “disintegration” (Fig. 1C; Refs. 10 and 20–26). The substrate for this reaction mimics one end of viral DNA joined to target DNA. Disintegration is the macroscopic reverse of the integration reaction: the adjacent 3′ hydroxyl of the target DNA attacks the phosphodiester bond joining viral and target DNA, releasing the model viral DNA end and concomitantly restoring the continuity of the target DNA. In order to interpret results of experiments using disintegration substrates, it is important to determine whether the arrangement of viral and target DNA near the active site is the same for the disintegration and integration reactions.

We used phosphorothioate-containing substrates to investigate the orientation of the reacting phosphodiester and the attacking nucleophile in end processing, integration, and disintegration. We found that substrates with an (R)-phosphorothioate at the reaction center were preferred by HIV-1 integrase as substrates for end processing and integration. In contrast, HIV-1 integrase preferentially catalyzed disintegration of substrates with an (S)-phosphorothioate at the reaction center. The stereospecificity of integrase in the three reactions provides information regarding the likely arrangement of the key substrate features in the active site and the relationship between viral and target DNA bound to the enzyme.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Reagents**

Integrase was purified as described previously (28). Oligonucleotides were purchased from Operon Technologies, Inc. (Emeryville, CA) and purified by electrophoresis on a 15% or 20% denaturing polyacrylamide gel prior to use. T4 polynucleotide kinase, T4 DNA ligase, and exonuclease I

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FIG. 1. Schematic depiction of the reactions catalyzed by HIV integrase. A, end processing; B, integration of the viral DNA end into target DNA; C, disintegration. The single active site is outlined by a dashed line. The DNA is represented by a ribbon structure, and the phosphate group undergoing attack in each case is represented by a solid circle. The location of the phylogenetically conserved CA dinucleotide is shown on the ribbon representing the 3' end of the viral DNA.

clease-free Klenow were purchased from New England Biolabs; modified T7 DNA polymerase (Sequenase version 2.0) was from U. S. Biochemical Corp.; snake venom phosphodiesterase (SV) and P1 phosphodiesterase (P1), [γ-32P]ATP, [α-32P]TTP, and [α-32P]GTP (specific activity of 3000 Ci/mol) from Amersham Pharmacia Biotech; and nucleotides and deoxynucleotides (5-μm particle size, 25 × 0.45 cm) using a gradient of 3–13% acetonitrile in 0.1 M acetic acid/imidazole, pH 6.5. The peak fractions were lyophilized under the same conditions for use as a standard. The 3'-end processing substrate to product. The reaction products were taken as soon as a single (racemic) phosphorothioate (see "Oligonucleotides"). The stereoisomers were separated by reverse phase HPLC on a C-18 column (200 μm particle size, 25 × 0.45 cm) using a gradient of 3–13% acetonitrile in 0.1 M acetic acid/triethylamine, pH 6.5. The dinucleotide AG was chromatographed under the same conditions for use as a standard. The early eluting peak contained a P1-resistant AG dinucleotide but was completely digested to mononucleotides by SV, while the late-eluting peak contained a P1-resistant AG dinucleotide but was completely digested by P1, indicating that the early peak contained an oligonucleotide with an (R)-phosphorothioate and the late peak contained an oligonucleotide with an (S)-phosphorothioate.

To make the end-processing substrate, each oligonucleotide containing a homogenous stereoisomer was phosphorylated at its 5' end with T4 polynucleotide kinase and cold ATP. After heat inactivation of T4 polynucleotide kinase, the phosphorothioate-containing oligonucleotide was annealed to VT4 and VB2. Ligase buffer and ligase were added to the annealed oligonucleotide with an (S)-phosphorothioate (27, 28). To the make the end-processing substrate, each oligonucleotide containing a homogenous stereoisomer was phosphorylated at its 5' end with T4 polynucleotide kinase and cold ATP. After heat inactivation of T4 polynucleotide kinase, the phosphorothioate-containing oligonucleotide was annealed to VT4 and VB2. Ligase buffer and ligase were added to the annealed oligonucleotide, and the reaction mixture was incubated overnight at 16 °C. Sequenase 2.0 and [α-32P]TTP were then used to fill in the 3' nucleotide of the VT4 strand ligated to the VT3 strand (see Fig. 5A). The phosphodiester substrate was made by annealing VT5 to VB1 and radiolabeling the 3' end of VT5 annealed to VX1. The 21-mer product was purified by denaturing gel electrophoresis, quantitated, and annealed to VB1.
Reaction Conditions

Disintegration—The reaction buffer for kinetic experiments performed with a dumbbell disintegration substrate (Fig. 2A) contained 20 mM HEPES, pH 7.5, 10 mM dithiothreitol, 10 mM MnCl₂, 0.05% Nonidet P-40, and 10 mM NaCl. Reactions were performed in duplicate and with 1 µM disintegration substrate and 200 nM integrase and were incubated at 37 °C. Aliquots were removed at specified times, quenched with formamide-determining gel-loading buffer, and substrates and products were separated by denaturing electrophoresis and quantitated using a Molecular Dynamics PhosphorImager. Results from duplicate experiments were averaged.

The disintegration reactions from which product was isolated for stereochemical analysis were carried out with 200 nM integrase and 100 nM substrate using the above buffer and conditions. Products were purified by denaturing gel electrophoresis, eluted in 0.5 M ammonium acetate plus 10 mM magnesium acetate, precipitated with ethanol, and resuspended in TE for digestion by the stereospecific phosphodiesterases.

Integration—Integration reactions were performed for stereochemical analysis in the same buffer used for the disintegration reaction by preincubating 20 nM radiolabeled viral end DNA and 300 nM integrase in 100 µl for 5 min at 37 °C and then adding 1 µM target DNA (either TT4 annealed to TB3 or TT5 annealed to TB4). After incubation for 1.5 h at 37 °C, reactions were quenched with formamide loading buffer, and the products purified by electrophoresis as described above for the disintegration product analysis.

3' End Processing—Reactions were carried out at 37 °C with 20 mM HEPES, pH 7.5, 10 mM DTT, 50 µg/ml bovine serum albumin, and 7.5 mM of either MgCl₂ or MnCl₂ and 300 nM dithiothreitol and 2.75 mM dithiothreitol and 2.75 mM sodium acetate, pH 5.2, at room temperature with 17 mM NaCl concentration in the reaction buffer for these reactions was 30 mM instead of 10 mM, which presumably accounts for the difference in the kinetics relative to the reactions shown in panel B (26).

RESULTS

Stereospecific Disintegration of Phosphorothioate Substrates—A dumbbell disintegration substrate, composed of a single oligonucleotide that folds on itself to form a structure mimicking one viral DNA end joined to target DNA (Fig. 2A; Ref. 22), was chemically synthesized with either a standard phosphodiester or a racemic phosphorothioate at the junction between viral and target DNA. The kinetics of disintegration of these two substrates were compared (Fig. 2B). Approximately 85% of the substrate with the standard phosphodiester bond at the junction was converted to product by 90 min, but only 45% of the substrate with the racemic phosphorothioate at the junction was converted to product even after 300 min. As chemically synthesized phosphorothioates are typically composed of the substrate or the preferred phosphorothioate stereoisomer. If the stereoisomers were present initially at a ratio near 1:1, then the disintegration rate of the preferred phosphorothioate stereoisomer was about 2-fold lower than that of the phosphodiester substrate.

To test and quantitate this preference for one stereoisomer, we purified the putative less preferred stereoisomer by isolating the residual substrate following overnight reaction of the dumbbell disintegration substrate with integrase. This material was then radiolabeled and used as a substrate in a second disintegration reaction (Fig. 2C). Parallel reactions were carried out with otherwise identical substrates containing a standard phosphodiester bond or a racemic phosphorothioate at the reaction center. The rate of disintegration of the phosphodiester substrate was at least 50-fold greater than that of the less preferred phosphorothioate stereoisomer. This value is a lower limit because of potential contamination of the less preferred stereoisomer substrate with either the phosphodiester substrate or the preferred phosphorothioate stereoisomer. If the substrate containing the racemic phosphorothioate was not substantially contaminated with phosphodiester substrate, and the stereoisomers were present initially at a ratio near 1:1, then the disintegration rate of the preferred phosphorothioate stereoisomer was about 2-fold lower than that of the phosphodiester substrate.
stereospecificity for \((S_p)\)-phosphorothioates (28), whereas SV has a high stereospecificity for \((R_p)\)-phosphorothioates (27).

A Y-mer disintegration substrate was constructed to determine the stereochemistry of the product of the disintegration reaction (Fig. 3A, see also “Y-mer Disintegration Substrates” under “Experimental Procedures”). After incubation at 37 °C for 4 h, approximately 65% of the phosphodiester substrate and 15% of the phosphorothioate substrate had been converted to product (data not shown).

The ligated target DNA strands from these disintegration reactions were isolated by gel electrophoresis and analyzed by digestion with P1 and SV. With the racemic phosphorothioate substrate, the ligated target DNA strand resulting from the disintegration reaction (Fig. 3B, lane 10) contained a dinucleotide that was resistant to digestion with P1 (lanes 11–14), but was completely digested to mononucleotides by SV (lanes 15–18). This cleavage pattern is expected for the \((R_p)\)-phosphorothioate isomer. The product originating from the phosphodiester substrate (Fig. 3B, lane 1) was completely digested by both P1 (lanes 2–5) and SV (lanes 6–9), under conditions identical to those used to digest the phosphorothioate-containing product. Control reactions with purified phosphorothioate stereoisomers validated the stereospecificity of these preparations of P1 and SV (see “Experimental Procedures”). Assuming that disintegration, like 3' end processing and integration (5), proceeds with inversion about the reactive phosphoryl group, integrase preferentially catalyzed disintegration of the substrate containing the \(S_p\) stereoisomer at the reaction center. Densitometry of the dinucleotide remaining in the final time point of the SV digestion (Fig. 3B, lane 18) gave a ratio of ~50 for the mononucleotide relative to the dinucleotide digestion product, suggesting a strong preference for the \((S_p)\)-phosphorothioate substrate over the \((R_p)\)-phosphorothioate substrate.

The \((R_p)\)-Phosphorothioate Stereoisomer Is the Preferred Target DNA Substrate for Integration—Phosphorothioate substrates were designed to monitor the stereochemistry of the products of the integration reaction (Fig. 4A). The phosphodiester bond immediately 3’ to the terminal adenosine residue of the viral DNA substrate was radiolabeled. The purified products from integration into either all-phosphodiester target DNA (Fig. 4B, lane 8) or the phosphorothioate-containing target DNA (lane 1) were digested separately with P1 or SV. The products of integration into the all-phosphodiester target DNA were completely digested to mononucleotides by both SV (lanes 9–11) and P1 (lanes 12–14). After extensive digestion of the products of integration into the phosphorothioate-containing target DNA with SV, residual AC and AG dinucleotides persisted (lanes 2–4), suggesting that these dinucleotides had the \(S_p\) stereoisomer expected from attack of the viral 3’ hydroxyl on \((R_p)\)-phosphorothioates 5’ to a C or G. The mononucleotides were the products expected from SV digestion of integration products in which the viral ends had joined 5’ to an A or T, residues without phosphorothioate substitution. Because the target oligonucleotide contained runs of up to six consecutive G and C nucleotides, each containing phosphorothioate linkages, SV digestion would also be expected to yield smaller quantities of products larger than a dinucleotide, as was observed (Fig. 4B, lanes 3 and 4). In contrast, the absence of an accumulation of dinucleotide and longer products with P1 digestion suggests that there was no significant integration reaction with the \((S_p)\)-phosphorothioates.

The \((R_p)\)-Phosphorothioate Stereoisomer Is the Preferred Substrate for 3' End Processing—Phosphorothioate-containing oligonucleotides mimicking a viral DNA end were designed to monitor the stereochemistry of the products of the end-processing reaction (Fig. 5A). To determine integrase’s stereospecificity for end processing, we compared the rates of end processing of stereochromically pure viral DNA end substrates containing either an \((S_p)\)- or \((R_p)\)-phosphorothioate at the reaction center. \((R_p)\)-Phosphorothioate, \((S_p)\)-phosphorothioate, and phos-
The observed stereospecificity of HIV-1 integrase in catalyzing end processing, integration, or disintegration of model substrates with phosphorothioates at the reaction center places new constraints on models for the orientation of reactants in the enzyme’s active site. A model for the arrangement of substrate features in the active site of HIV integrase, based on these results and previous observations, is illustrated in Fig. 6. This model begins with three assumptions. 1) The transition state is a trigonal bipyramid, with the nucleophile and leaving groups occupying the apical positions for an in-line attack and the other three, equatorial substituents coplanar with the central phosphorus, as expected from chemical and enzymological precedents (32, 33). 2) Integrase uses a single active site to catalyze end processing, integration, and disintegration, as suggested by mutational and structural results (6–13). 3) Certain specific interactions in the active site are the same for all three reactions (see below). In developing this model, we first consider groups occupying the apical positions of the trigonal bipyramidal transition state, basing our discussion on previous results. We then turn to results from the present study that pertain to the placement of groups in the equatorial positions of the transition state.

Both in vivo and in vitro results suggest that the phylogenetically conserved CA/TG dinucleotide pair adjacent to the cleavage site on the viral DNA end is a crucial feature for recognition of viral DNA by integrase. In vitro, changing the phylogenetically conserved CA/TG dinucleotide pair adjacent to the cleavage site in a model substrate results in up to a 50-fold greater rate for the $S_p$ stereoisomer substrate, when Mn$^{2+}$ was used as the metal ion cofactor (Fig. 5B). The rate of end processing for the phosphodiester substrate was only slightly higher than the rate observed for the ($R_p$)-phosphorothioate substrate. The same stereospecificity was observed with Mg$^{2+}$ as the metal ion cofactor (Fig. 5C); the ($R_p$)-phosphorothioate substrate was processed at a rate 40-fold higher than that observed with the ($S_p$)-phosphorothioate substrate, and the phosphodiester substrate was processed slightly faster than the ($R_p$)-phosphorothioate substrate.

Integrase also preferred the ($R_p$)-phosphorothioate substrate for end processing in reactions carried out in the presence of 7.5 mM MnCl$_2$ and 10% glycerol (data not shown). It has been previously shown that end processing can give three chemically distinct dinucleotide products, depending on the nucleophile used (5, 31). When Mg$^{2+}$ is included as the divalent cation, water is the primary nucleophile, and the main product is the hydrolysis product, a linear GT dinucleotide. However, in the presence of Mn$^{2+}$, a variety of nucleophiles can be used, including glycerol, generating the dinucleotide (GT) product linked at its 5’ end to glycerol, or the terminal 3’ hydroxyl of the viral DNA, producing a 3’-5’ cyclic dinucleotide. Furthermore, the ($R_p$)-phosphorothioate substrate and the phosphodiester substrate yielded similar proportions of cyclic product (data not shown). Thus, the substitution of sulfur for a non-bridging oxygen did not appear to alter the propensity of the terminal 3’ hydroxyl to reach and attack the reactive subterminal viral phosphodiester relative to attack by water. In contrast, the proportion of product formed from the use of glycerol as a nucleophile was 4-fold lower for the ($R_p$)-phosphorothioate substrate than for the phosphodiester substrate (Fig. 5D). Since sulfur is a much poorer hydrogen bond acceptor than oxygen, this result suggests that hydrogen bonding between the cis-hydroxyl of the glycerol and the oxygen of the reactive phosphate helps to position the glycerol for nucleophilic attack. Such an interaction could account for the preference for cis-diols relative to simple alcohols as nucleophiles in the end-processing reaction (31).

### DISCUSSION

The observed stereospecificity of HIV-1 integrase in catalyzing end processing, integration, or disintegration of model substrates with phosphorothioates at the reaction center places new constraints on models for the orientation of reactants in the enzyme’s active site. A model for the arrangement of substrate features in the active site of HIV integrase, based on these results and previous observations, is illustrated in Fig. 6. This model begins with three assumptions. 1) The transition state is a trigonal bipyramid, with the nucleophile and leaving groups occupying the apical positions for an in-line attack and the other three, equatorial substituents coplanar with the central phosphorus, as expected from chemical and enzymological precedents (32, 33). 2) Integrase uses a single active site to catalyze end processing, integration, and disintegration, as suggested by mutational and structural results (6–13). 3) Certain specific interactions in the active site are the same for all three reactions (see below). In developing this model, we first consider groups occupying the apical positions of the trigonal bipyramidal transition state, basing our discussion on previous results. We then turn to results from the present study that pertain to the placement of groups in the equatorial positions of the transition state.

Both in vivo and in vitro results suggest that the phylogenetically conserved CA/TG dinucleotide pair adjacent to the cleavage site on the viral DNA end is a crucial feature for recognition of viral DNA by integrase. In vitro, changing the phylogenetically conserved CA/TG dinucleotide pair adjacent to the cleavage site in a model substrate results in up to a
In vivo, integration of viral genomes in which the conserved CA/TG dinucleotide pair has been altered by mutation is impaired by a factor of $10^5$. Since the CA/TG dinucleotide pair is critical for all three reactions, it is reasonable to assume that this substrate feature is bound in the same specific site for end processing, integration, and disintegration. This site is depicted in yellow in Fig. 6.

In the transition state for phosphoryl transfer, the nucleophile and the leaving group typically occupy the apical positions. As noted above, previous results have strongly suggested that the phylogenetically conserved viral CA/TG base pairs are in bold type, and the arrowhead indicates the site of processing by integrase. The phosphodiester substrate was prepared by 3' end-labeling a top strand lacking the final T with [α-32P]TTP. B and C, end-processing kinetics for viral DNA end substrates containing an (Rp)-phosphorothioate (A), (Sp)-phosphorothioate (○), or phosphodiester (○) at the cleaved position. Either 7.5 mM MnCl2 (panel B) or 7.5 mM MgCl2 (panel C) was included as the divalent metal ion cofactor for the end-processing reactions. D, substitution of a sulfur atom for a non-bridging oxygen has an unfavorable effect on the ability of glycerol to serve as the nucleophile for 3' end processing of a model viral DNA end substrate. Reactions were carried out in the presence of 7.5 mM MnCl2 and 10% glycerol. The graph shows the time course of accumulation of the glycerol-linked dinucleotide product from reactions with the phosphodiester-containing substrate (○), or an (Rp)-phosphorothioate substrate (○), as well as the time course for accumulation of all end-processing products (including the hydrolysis product, the glycerolysis product, and the cyclic product) from the same reactions with a phosphodiester-containing substrate (●), or an (Rp)-phosphorothioate substrate (▲).

According to the proposed model, the “blue” apical position would then be occupied by water or an alcohol hydroxyl acting as the nucleophile in end processing, or a target DNA 3' hydroxyl acting as the nucleophile or the leaving group in disintegration and integration, respectively.

We now consider the groups that occupy the equatorial positions. The stereospecificity observed for phosphorothioate stereoisomers in the end-processing, integration, and disintegration reactions suggest that, in each of these reactions, the active site interacts with one of the non-esterified phosphoryl oxygen atoms in manner that discriminates against a substituted sulfur atom. Fig. 6 presents the simplest model to account for these observations, in which a single site, depicted in pink, interacts with a non-esterified oxygen atom, but cannot readily accommodate a sulfur atom. Once the occupants of the two apical positions and one of the three equatorial positions in the transition state are specified, the observed stereospecificities of the three integrase-catalyzed reactions dictate the ar-
rangements of the remaining substituents. Somewhat surprisingly, the results strongly imply that the site occupied by the target DNA extending 3' from the reaction center (indicated in purple in Fig. 6) is not the same as the site that accommodates the 3' terminal dinucleotide of the viral DNA end in the end-processing reaction (indicated in green in Fig. 6). The basis for this specific arrangement is not known. One possibility is that the site that binds the 3' terminal dinucleotide in the end-processing reaction (green) interacts favorably with a single strand of DNA in a configuration that it can only adopt when not base paired. If double-helical DNA cannot be accommodated in this site (green), then the target DNA extending 3' from the reaction center would be constrained to occupy this position. DNA targets can vary widely with respect to the size and structure of the features 3' to the reaction center (22). Thus, this (purple) site may better be viewed as a space that can accommodate this portion of the target DNA rather than a site that provides crucial specific binding interactions. Although results of previous photo-cross-linking studies of the integrase-DNA complex were not explicitly used in developing the model shown in Fig. 6, the resulting model is entirely consistent with the architecture of the integrase-DNA complex inferred from those cross-linking studies (43).

Macroscopically, disintegration is the reverse of the integration reaction. Moreover, the substrate specificity of the reactions is homologous (22, 26, 35, 44). Integrase’s preference for opposite phosphorothioate stereoisomers for integration and disintegration provides further support for a reciprocal relationship between the two reactions and for a model in which the local arrangement of viral and target DNA in the active site is the same for the two reactions.

Enzymes in the integrase/transposase family of polynucleotidyl transferases all apparently catalyze cleavage and joining of the transposable element DNA by similar mechanisms (5, 45, 46). Many of these enzymes, including the VDJ recombinase, the bacteriophage Mu transposase, HIV-1 integrase, and murine leukemia virus integrase, appear to disrupt base pairing in the vicinity of the reaction center in their otherwise double-helical substrates, and these enzyme-induced disruptions of the double-helical structure of their substrates can facilitate catalysis in vitro (14–19, 47, 48). This local disruption of DNA...
structure appears to be a common means by which these enzymes manage to use the same active site for donor cleavage and joining. It will now be interesting to investigate whether the evolutionary conservation of structural and mechanistic features of the enzymes in this family will extend to the arrangement of the analogous DNA substrates in their active sites.

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