to 4 weeks in medium supplemented with geneticin G418 [(Gibco-BRL, Gaithersburg, MD), at 400 μg/ml for 1 week; 200 μg/ml, 2 to 3 weeks] and then screened for human IκB-α by immunoblotting. The results with each transfected IκB-α species were confirmed with independently transfected cell clones.
9. V. Bours *et al.*, *Mol. Cell. Biol.* **12**, 685 (1992); G. Franzoso *et al.*, *Nature* **359**, 339 (1992); V. Bours *et al.*, *Cell* **72**, 729 (1993).
10. We constructed the ΔN and ΔC IκB-α mutants by cloning into PMT2T (*9*) the 1296–base pair (bp) Xho I–Eco RI fragment and the 921-bp Eco RI–Xmn I fragment, respectively, of the human gene (*15*). Point mutants of IκB-α were obtained in a Bluescript plasmid (Stratagene, La Jolla, CA) containing nucleotides 1 to 1550 of IκB-α complementary DNA (*15*) by site-directed mutagenesis [P. Bressler *et al.*, *J. Virol.* **67**, 288 (1993)]; the mutants were then cloned into PMT2T as 1550-bp Eco RI fragments encoding full-length IκB-α.
11. To permit detection of the newly phosphorylated IκB-α in this experiment, we included phosphatase inhibitors during cell extraction (*2, 5*). To enhance detection, we trapped the phosphorylated intermediate by blocking proteolysis with calpain inhibitor I (*2*).
12. K. Brown, S. Gerstberger, L. Carlson, G. Franzoso, U. Siebenlist, unpublished data.
13. These include all four other potential phosphoacceptor sites within the NH_2-terminal 89 amino acids (residues Tyr^{42}, Ser^{63}, Thr^{71}, and Ser^{76}) and other sites throughout the ankyrin and COOH-terminal domain. Of note, mutation of the protein kinase C consensus site (residues Ser^{262} and Thr^{263}) (*15*) did not prevent activation of NF-κB (*12*).
14. Proteolytic mapping experiments confirmed that induced phosphorylation of IκB-α occurred at a site or sites that are NH_2-terminal to the ankyrin domain (*12*). Thus together with the observation that mutations of all other potential phosphorylation sites within the NH_2-terminal domain of IκB-α did not block signaling indicates that the possibility that the Ser^{32} and Ser^{36} mutations act directly at one or both of these sites to block phosphorylation.
15. S. Haskill *et al.*, *Cell* **65**, 1281 (1991).
16. S. Rogers, R. Wells, M. Rechsteiner, *Science* **234**, 364 (1986).
17. A similar ΔC mutant has previously been shown to inhibit DNA binding of NF-κB in vitro [E. N. Hatada, M. Naumann, C. Scheidereit, *EMBO J.* **12**, 2781 (1993)].
18. Two-dimensional gel electrophoresis was performed as described [P. H. O'Farrell, *J. Biol. Chem.* **250**, 4007 (1975)] by Kendrick Labs, Inc. (Madison, WI). The gels of ^{35}S-labeled murine IκB-α revealed constitutive phosphoisoforms analogous to those of human IκB-α. These experiments also showed that the murine IκB-α was phosphorylated in response to signals (shown by a shift in charge toward negative pH in the first dimension) and that the induced phosphorylation of this species resulted in only a minor shift in apparent size (*12*). Analyses of the ΔN and mt^{36} mutants yielded results similar to those obtained with mt^{32} (*12*). Phosphatase treatment resulted in a single spot (*12*). Endogenous IκB-α from human cells was indistinguishable from exogenously expressed human IκB-α on these gels.
19. R. de Martin *et al.*, *EMBO J.* **12**, 2773 (1994).
20. It remains to be determined if one or both serines are phosphorylated. Mutation of one of these closely spaced serines may block phosphorylation of the other; even if both serines are phosphorylated, their phosphorylation may be linked.
21. We thank K. Kelly and A. S. Fauci for review of the manuscript, A. S. Fauci for continued support, and Y. Ward for expert help and advice.

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Requirement of RNA Polymerase III Transcription Factors for in Vitro Position-Specific Integration of a Retroviruslike Element

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The yeast retroviruslike element Ty3 inserts at the transcription initiation sites of genes transcribed by RNA polymerase III (Pol III). An in vitro integration assay was developed with the use of Ty3 viruslike particles and a modified *SUP2* tyrosine transfer RNA ($tRNA^{Tyr}$) gene target. Integration was position-specific and required Ty3 integrase, Pol III transcription extract, and a transcriptionally competent tRNA gene. Use of individual transcription factor (TF) IIIB–, TFIIIC-, and Pol III–containing fractions showed that TFIIIB and TFIIIC, together, were sufficient for position-specific Ty3 integration, but not for transcription. This report demonstrates that in vitro integration of a retroelement can be targeted by cellular proteins.

Integration of replicated DNA into the host genome is essential for retroviruses and retrotransposons. Integration is nonrandom, but the mechanisms that determine the positions of integration remain poorly defined [reviewed in (*1, 2*)]. DNA sequence, chromatin structure, and the presence of host cell proteins can influence target site selection. On the basis of some in vivo studies, retroviral integration appears to occur preferentially into regions that contain deoxyribonuclease I (DNAase I) hypersensitive sites and regions that are transcriptionally active. A study of avian leukosis virus integration in vivo suggested that most or all of the cellular genome was accessible to integration machinery, but that regional and site preferences existed (*3*). In a series of selected integrations, the yeast retroviruslike element Ty1 integrated with a preference for the 5′ portions of the three RNA Pol II–transcribed genes examined (*4*). In addition, unselected transposition of Ty1 into chromosome III revealed an insertion bias for regions near tRNA genes and outside of open reading frames; this pattern was not observed in vitro with a naked DNA substrate (*5*). These findings have implicated host DNA-associated proteins in integration site selection.

DNA structure influences retroviral integration site selection. This was suggested by a study that identified in vivo integration sites in DNA fragments containing bends (*6*). In addition, in vitro data indicate that integration occurs preferentially into particular DNA structures. Integration mediated by human immunodeficiency virus (HIV) pre-integration complexes or purified integrase (IN) occurred nonrandomly into unmodified, naked DNA and preferentially into runs of CpG modified by 5-methylation of cytosine (*7*). With nucleoprotein complexes of murine leukemia virus (MLV), integration into naked DNA also occurred nonrandomly and more frequently into the exposed major groove of DNA assembled into nucleosomes (*8*). Moreover, DNA positions predicted to be distorted within the nucleosome were used preferentially for integration mediated by HIV IN (*9*). Preferred integration into static or induced bends that were not associated with nucleosomes occurred with both MLV and HIV IN (*10*).

Ty3, a retroviruslike element in *Saccharomyces cerevisiae*, integrates solely upstream of genes transcribed by Pol III. The gene-proximal joint in the host DNA occurs within one to three nucleotides of the transcription initiation site (*11, 12*). On the basis of in vivo experiments, it has been suggested that integration is mediated by a protein-protein interaction between the Ty3 integration complex and the Pol III transcription complex. To identify cellular factors required for Ty3 integration, we developed an in vitro assay. Ty3 viruslike particles (VLPs), analogous to retroviral core particles, were isolated as a complex fraction containing Ty3 RNA, replicated DNA, and the mature Ty3 proteins, including IN (*13*). The target plasmid, pDLC374 (Fig. 1A), was used previously as a target for Ty3 transposition in vivo monitored by genetic selection (*12*) or by a polymerase chain reaction (PCR) assay (*14*). This plasmid contained the yeast *HIS3* gene, a 2-μm plasmid origin of replication, and a modified version of the *SUP2* $tRNA^{Tyr}$ gene cloned into pIBI20. To reconstitute the natural genomic targets of Ty3, we prepared a Pol III transcription extract from yeast cells (*15*). The BioRex70 500 mM NaCl fraction (BR500) from this preparation was used together with Ty3 VLPs and the tRNA gene target plasmid for in vitro integration reactions (*16*). DNA was isolated from each reaction and amplified by PCR with primers

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complementary to the *HIS3* gene (primer set 284-285) to quantitate the amount of pDLC374, or with primers complementary to Ty3 and the tDNA (primer set 278-279) to detect position-specific integration (Fig. 1A) (*17*). PCRs were performed previously with each primer set and either pDLC374 or pTM42, a pDLC374 derivative which has a Ty3 insertion in divergent orientation immediately upstream of the transcription initiation site of the tRNA gene (*14*). A 474–base pair (bp) product from the 278-279 PCR was detected for pTM42 but not for pDLC374. The 240-bp product from the *HIS3* primers (284-285 PCR) was detected for both plasmid templates.

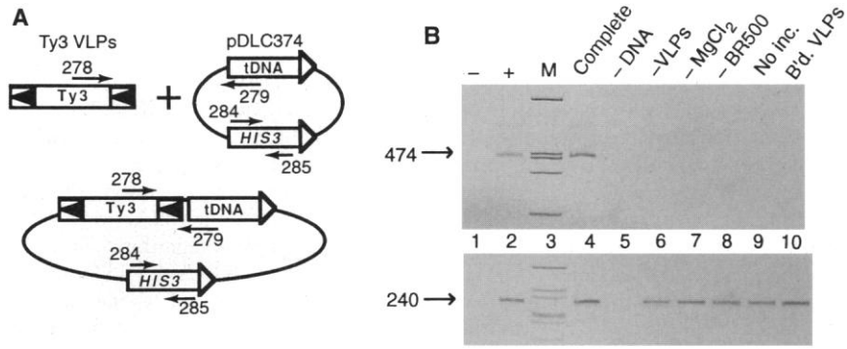

Fig. 1. Ty3 VLPs mediate integration in vitro. (**A**) Diagram for detection of in vitro integration of Ty3 by a PCR assay. PCR primers and their positions in the Ty3 and the modified *SUP2* tRNA^{Tyr} sequences are shown. If Ty3 integration occurs into target plasmid pDLC374 upstream of the tRNA gene, in the divergent orientation, and at the insertion site used most frequently in vivo (*12*), primer 279 spans the Ty3-tDNA junction. PCR amplification of the resulting integration product with primer set 278-279 produces a 474-bp product. Primer set 284-285 amplifies a 240-bp fragment in the *HIS3* gene. (**B**) In vitro integration. Integration reactions were performed (*16*) and analyzed by PCR (*17*). The lane numbers refer to both upper and lower panels. Upper panel, products from 278-279 Ty3-specific PCR with 8 ng of template; lower panel, products from 284-285 *HIS3*-specific PCR with 0.1 ng of template (lanes 1 and 5, no DNA). Lane 4, standard integration reaction. Lane 7, $MgCl_2$ was omitted and 1 mM EDTA added. Other omissions from the integration reaction were as indicated above each lane. No inc. indicates that the 30°C incubation was omitted, and B'd. indicates that the VLPs were boiled for 10 min. In all figures of PCR products, M indicates size markers (λ DNA digested with Pst I), and the – and + signs refer to control reactions for the PCR assay (no template and 0.1 ng of pTM42, respectively). PCR product sizes are indicated to the left in base pairs.

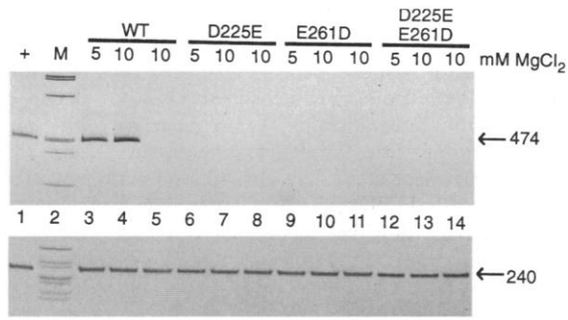

Fig. 2. Integration requires VLPs with functional IN. VLPs were isolated from yeast cells expressing wild-type (WT) Ty3 or Ty3 with mutations in IN: D225E, E261D, or both. Mutant VLPs were used in standard integration reactions in parallel with WT VLPs as follows: WT, lanes 3 to 5; D225E, lanes 6 to 8; E261D, lanes 9 to 11; D225E + E261D, lanes 12 to 14. Integration reactions included 5 mM $MgCl_2$ (lanes 3, 6, 9, and 12), 10 mM $MgCl_2$ (lanes 4, 7, 10, and 13), or 10 mM $MgCl_2$ and boiled VLPs (lanes 5, 8, 11, and 14). The lane numbers refer to both upper and lower panels. Upper panel, 278-279 PCR with 6 ng of DNA; lower panel, 284-285 PCR with 0.1 ng of DNA. PCR product sizes are indicated to the right in base pairs.

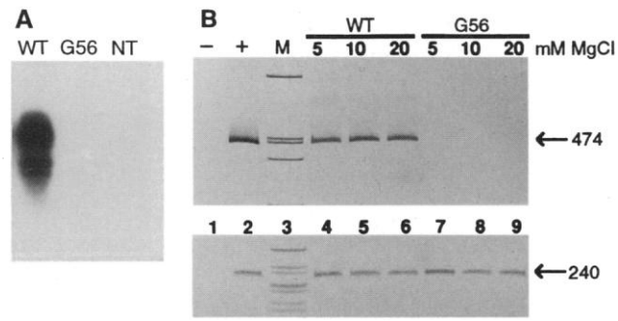

Fig. 3. In vitro integration depends on the presence of BR500 transcription extract. (**A**) Transcription assays. The templates for transcription were 400 ng of pDLC374 (WT) or the G56 mutant (G56). NT indicates no template. Assays were performed as in (*15*) except that the reactions had 250 μM adenosine triphosphate, uridine triphosphate, and cytidine triphosphate; 15 μM guanosine triphosphate (GTP); and 10 μCi [α-^{32}P]GTP (3000 cpm/mmol, Amersham) and were incubated at 30°C. There was 100 μg of BR500 protein in each reaction. (**B**) Integration reactions. Standard integration reactions (*16*) were performed with WT (lanes 4 to 6) and G56 (lanes 7 to 9) targets in the presence of 5, 10, or 20 mM $MgCl_2$. The lane numbers refer to both upper and lower panels. Upper panel, 278-279 PCR with 2 ng of DNA; lower panel, 284-285 PCR with 0.1 ng of DNA. PCR product sizes are indicated to the right in base pairs.

In vitro integration reactions were performed with the standard conditions as described (*16*) or with variations in order to identify essential reaction components (Fig. 1B). When the standard integration reaction conditions were used, the 474-bp 278-279 PCR product was observed, which indicated that integration occurred upstream of the tRNA gene. Integration required target DNA, VLPs, and a divalent cation. No integration occurred if the incubation time was 0 min or if the VLPs were boiled for 10 min before being added. There was no detectable integration in the absence of BR500 transcription extract, suggesting that a cellular protein or proteins were required for the position-specific integration. Differences in the amount of the 474-bp product were not the result of variation in the amount of DNA template for the PCR as demonstrated by equivalent amounts of the control 240-bp *HIS3* product. The 278-279 PCR products of several integration samples were compared with the 278-279 PCR products of a serial dilution of pTM42 DNA in pDLC374 DNA. On the basis of this comparison, integration was estimated to occur into less than 5% of the target DNA.

To verify that integration was the result of Ty3 IN activity, we isolated VLPs from cells expressing Ty3 with mutations in the IN protein. The conserved motif Asp-$X_{(39–58)}$-Asp-$X_{(35)}$-Glu occurs within the putative catalytic domain of IN in retroviruses and retroviruslike elements, and the invariant Asp and Glu residues are required for IN catalysis in vitro (*18*) and in vivo (*19*). Mutations engineered in Ty3 IN by site-directed mutagenesis changed the second Asp residue of this motif (amino acid position 225) (*20*) to Glu, and the Glu (amino acid position 261) to Asp. The mutations were introduced separately and in combination. The IN mutants produced wild-type amounts of mature Ty3 proteins and replicated DNA, but they did not transpose in vivo (*21*). When used in standard integration reactions in parallel with wild-type VLPs, the mutant VLPs were unable to catalyze integration (Fig. 2). Thus, in vitro, as in vivo, position-specific integration required Ty3 IN activity.

The dependence of the integration reaction on the presence of the BR500 extract suggested that integration in vitro depended on the ability of the tDNA to form a tran-

scription complex, as had been observed in vivo (*12*). To test this directly, we introduced a mutation into the tDNA promoter. Two internal promoter elements (box A and box B) in tRNA genes mediate the binding of the Pol III transcription factors TFIIIC and TFIIIB. TFIIIC binds first to the box A and box B sequences, then mediates the binding of initiation factor TFIIIB (*22*). We introduced a mutation by site-directed mutagenesis at the position in the modified *SUP2* $tRNA^{Tyr}$ gene corresponding to position 56 in the wild-type $tRNA^{Tyr}$ molecule. This mutation (G56) altered the first absolutely conserved cytosine in the box B promoter element, replacing it with guanine. The G56 mutation causes a decrease in the binding of TFIIIC to the *SUP4* $tRNA^{Tyr}$ gene in vitro to approximately 0.3% of wild-type amounts (*23*), which results in a decrease in transcription of *SUP4* $tRNA^{Tyr}$ in vivo to 5% of wild-type amounts (*24*). This mutation inactivates the *SUP2* $tRNA^{Tyr}$ gene as a target for Ty3 integration in vivo (*12*). In vitro, the modified *SUP2* $tRNA^{Tyr}$ gene with a wild-type box B was transcribed by the BR500 extract, but the G56 mutant was not (Fig. 3A). The G56 mutant was not used as a target for position-specific integration (Fig. 3B, lanes 7 to 9). These data showed that in vitro, as in vivo, integration required an intact box B. This result is consistent with the dependence of Ty3 integration on a Pol III transcription factor (or factors), Pol III, or both.

Integration into the modified *SUP2* $tRNA^{Tyr}$ gene with a wild-type or G56 mutant box B was also examined by performing the PCR with primers that did not anneal to the Ty3-target junction (*21*). With this integration position-independent primer set, a product of the predicted size for Ty3 integration immediately upstream of the tRNA gene was formed when the target was wild-type, but not when it was the G56 mutant. It is unlikely that our system can detect single insertions; thus, we cannot address the possibility that some insertions occur nonspecifically. However, the major site of in vitro integration detected with these primers was immediately upstream of the transcription initiation site.

To determine which Pol III transcription components were required for integration, we separated the BR500 extract on a DEAE Sephadex column into TFIIIB-, TFIIIC-, and Pol III–containing fractions (*15*). The fractions were tested in transcription reactions with pDLC374 (Fig. 4A). In vitro transcription activity was dependent on the presence of all three fractions. Transcription was not observed with the G56 mutant template or in the absence of template. The DEAE fractions were added to integration reactions in all combinations (Fig. 4B). No single fraction was sufficient for integration. Integration was observed when all three fractions were present, as expected, but also when only TFIIIC and TFIIIB were added. When Pol III was included, the amount of integration was lower. This result was obtained in duplicate experiments for three preparations of extract. Because TFIIIC is required to load TFIIIB onto the tDNA, these data do not address whether the role of TFIIIC in integration is direct or indirect, that is, as a loading factor.

The simplest model to explain the position-specific integration of Ty3 is that a protein-protein interaction occurs between some component of TFIIIB, either alone or together with TFIIIC, and the Ty3 integration complex. A tRNA gene that had reduced transcriptional activity as a result of mutations at the initiation site was competitive as a target for integration in vivo (*12*). That result suggested that transcription is not rate-limiting for transposition and is consistent with the in vitro result which showed that Pol III is not required for integration. The decrease in integration in the presence of Pol III may be the result of a competition between Pol III and the integration complex for contact with the TFIII(C+B)-tDNA complex.

In addition to a protein-protein interaction, there may be a DNA structural component to Ty3 integration. TFIIIC and TFIIIB bend the tDNA upon binding to the promoter elements and 5′ flanking region (*25*). The data are consistent with the center of the bend being positioned near the transcription start site. In addition, the initiation site is DNAase I–hypersensitive (*15*). DNA distortion in this region may facilitate access or activity of the Ty3 integration complex. Kinked and unwound regions of DNA appear to be used preferentially for retroviral integration, and distortion of DNA is an inherent feature in the activity of other endonucleases, for example Eco RI (*26*), and recombinases, for example FLP (*27*).

Ty3 is the most specific among well-studied retroelements in its pattern of integration, yet characterization of its mechanism of integration may elucidate the basis of more subtle integration patterns of retroviruses and other retroviruslike elements. The results presented here show the dependence of Ty3 position-specific integration on Pol III transcription factors. Retroviral and Ty1 integrations display some preference for transcribed regions and transcription initiation regions, respectively, and thus could be facilitated by interaction with transcription factors. HIV IN interacts with a human gene product (InI1) which has a region similar to one in the yeast transcription factor Snf5p and stimulates the joining activity of IN in vitro (*28*). Targeting of Ty3 preintegration complexes by transcription factors is compatible with a retroviral mechanism of integration and suggests a basis for the observed insertion of retroviruses into preferred positions in host genomes.

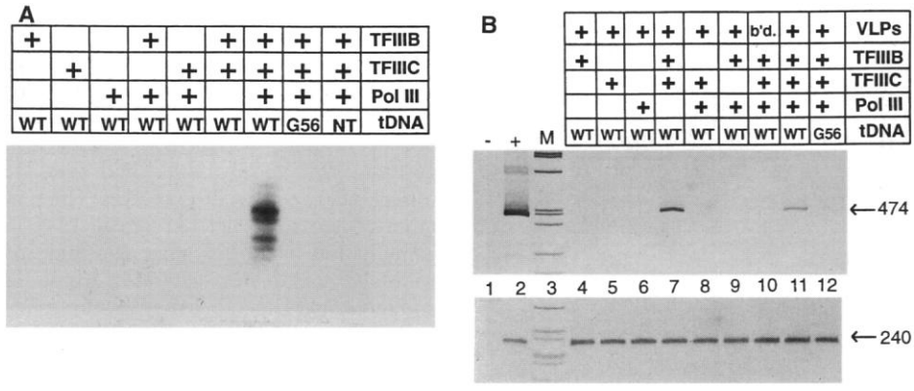

Fig. 4. In vitro integration depends on the presence of TFIIIC- and TFIIIB-containing fractions. (**A**) Transcription assays. Reactions were as described in the legend for Fig. 3A except 9 μl of each TFIIIC-, TFIIIB-, or Pol III–containing fraction (as indicted by the + sign) replaced the BR500 extract. (**B**) Integration reactions. Standard integration reaction conditions (*16*) were used except 9 μl of each TFIIIC-, TFIIIB-, or Pol III–containing fraction replaced the BR500 extract. B'd. indicates that VLPs were boiled for 10 min before being added. The lane numbers refer to both upper and lower panels. Upper panel, 278-279 PCR with 2 ng of DNA; lower panel, 284-285 PCR with 0.1 ng of DNA. PCR product sizes are indicated to the right in base pairs.

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16. The VLPs were isolated as described (*13*) with a sucrose step gradient. The VLP protein from 1 liter of cells (~330 μg) was resuspended in 40 μl of buffer [13.5 mM KCl, 9 mM Hepes (pH 7.8), 4.5 mM $MgCl_2$, and 10% glycerol] and kept on ice until used. A standard integration reaction consisted of 2 μl of resuspended VLP protein, 400 ng of pDLC374 (0.075 pmol), 20 mM Hepes (pH 7.8), 65 mM KCl, 1 mM dithiothreitol, 5 μl of BR500 transcription extract (at a final concentration of 1.8 to 2.0 mg/ml), and 10 mM $MgCl_2$ in a 50-μl reaction volume. If $MnCl_2$ replaced $MgCl_2$ in the reaction, integration was severely decreased (*21*). Per reaction, the VLPs provided 0.18 to 0.34 pmol of IN and 0.03 to 0.07 pmol of replicated Ty3 DNA (determined by comparison with standards). Reaction components were assembled on ice then incubated at 30°C for 30 min. Reactions were stopped by addition of proteinase K to a final concentration of 0.2 mg/ml, SDS to 0.2%, and EDTA (pH 8.0) to a concentration twice that of $MgCl_2$ in the integration reaction, then incubated at 37°C for 30 min. When necessary, $MgCl_2$ was added with the stop buffer to equalize the concentration of $MgCl_2$ in different samples.
17. The DNA from each integration reaction was isolated and quantitated by fluorometric analysis with a Mini TKO 100 DNA Fluorometer (Hoeffer Scientific). The PCR conditions for amplification of *HIS3* were similar to those described previously (*14*) except that the reactions with primer set 284-285 included 0.05 mM deoxynucleotide triphosphates (dNTPs) and 0.1 ng of template DNA and 18 cycles were run. The PCR conditions for detection of integration were similar to those described previously (*14*) except that the reactions with primer set 278-279 included 0.5 mM dNTPs, 3.5 mM $MgCl_2$, and 1 to 10 ng of template DNA (depending on the linear range, determined for each set of integration reactions). Primer 278 is complementary to nucleotides 444 to 418 of Ty3 on the transcribed strand. Primer 279 is complementary to the modified *SUP2* $tRNA^{Tyr}$ gene at nucleotides +25 to −5 on the transcribed strand (+1 corresponds to the transcription start site) and its 3′-most two nucleotides are complementary to Ty3 nucleotides 1 and 2 on the minus strand. The 278-279 PCR detected as little as 1×10^{-5} ng of pTM42 in the presence of 2 ng of pDLC374. The PCR products were analyzed by native polyacrylamide gel electrophoresis on 8% gels and visualized by transillumination with ultraviolet light after staining with ethidium bromide. Photographs were taken with Polaroid film (type 55), and the negatives were used for the figures presented here. For 284-285 PCRs, the negative was scanned with an LKB Ultroscan II Laser Densitometer (Pharmacia) to determine the band intensity for quantitation before the 278-279 PCR.
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29. We thank T. M. Menees for communicating results before publication and for helpful discussions. We also thank H.-P. Muller, H. E. Varmus, G. V. Kalpana, and S. P. Goff for communicating results before publication. Supported by NIH grant 33281 to S.B.S.

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Glutamate Receptor RNA Editing in Vitro by Enzymatic Conversion of Adenosine to Inosine

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RNA encoding the B subunit of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) subtype of ionotropic glutamate receptor (GluR-B) undergoes a posttranscriptional modification in which a genomically encoded adenosine is represented as a guanosine in the GluR-B complementary DNA. In vitro editing of GluR-B RNA transcripts with HeLa cell nuclear extracts was found to result from an activity that converts adenosine to inosine in regions of double-stranded RNA by enzymatic base modification. This activity is consistent with that of a double-stranded RNA-specific adenosine deaminase previously described in *Xenopus* oocytes and widely distributed in mammalian tissues.

The editing of mRNA transcripts is an important mechanism for augmenting the flexibility of eukaryotic gene expression (*1*). The A→G conversion seen in mRNAs encoding glutamatergic ion channel subunits exemplifies the functional relevance of this posttranscriptional modification (*2*). Glutamate receptors of the AMPA subtype are generated by the assembly of GluR-A, -B, -C, and -D subunits into homo- and heteromeric channels (*3*). A single positively

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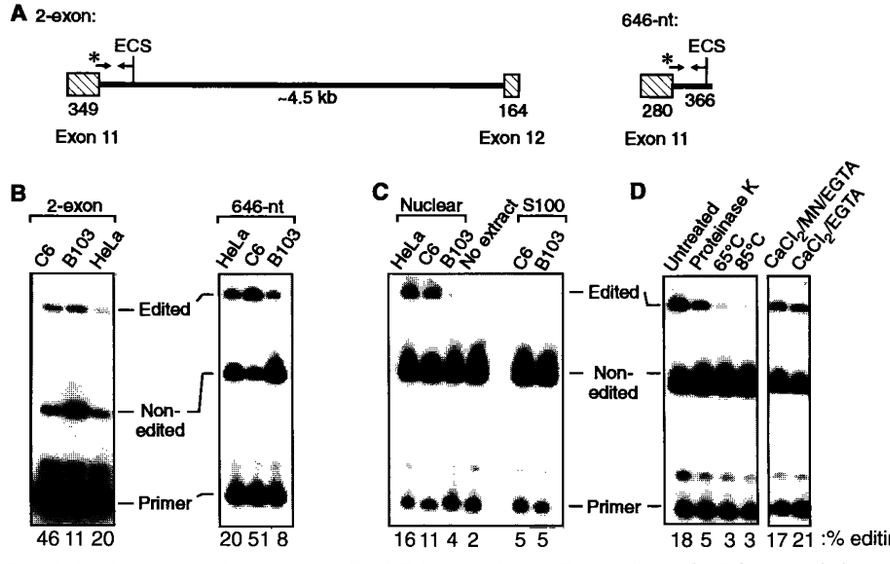

Fig. 1. In vivo and in vitro editing of GluR-B transcripts. (**A**) Schematic of GluR-B transcription units indicating the Q/R site (asterisk) and inverted repeat (arrows). (**B**) Primer-extension analyses of RNA from cells permanently transfected with GluR-B transcription units. (**C**) Primer-extension analyses of the 646-nt in vitro reaction products with nuclear and S100 extracts from the indicated cells. (**D**) Primer-extension analyses of in vitro reaction products generated in HeLa cell nuclear extracts pretreated as indicated. MN, micrococcal nuclease. The numbers at the base of each gel indicate the percent of A→G conversion (editing efficiency).