Insulin Secretion from Pancreatic B Cells Caused by L-Arginine–Derived Nitrogen Oxides

HARALD H. H. W. SCHMIDT,* TIMOTHY D. WARNER, KUNIO ISHII, HONG SHENG, FERID MURAD

L-Arginine causes insulin release from pancreatic B cells. Data from three model systems support the hypothesis that L-arginine–derived nitrogen oxides (NOs) mediate insulin release stimulated by L-arginine in the presence of D-glucose and by the hypoglycemic drug tolbutamide. The formation of NO in pancreatic B cells was detected both chemically and by the NO-induced accumulation of guanosine 3′,5′-monophosphate. N^G-substituted L-arginine analogs inhibited the release of both insulin and NO. Protein immunoblot and histochemical analysis with antiserum to type I NO synthase suggest that the formation of NO in pancreatic B cells is catalyzed by an NADPH- (reduced form of nicotinamide adenine dinucleotide phosphate), Ca^{2+}/calmodulin-dependent type I NO synthase of about 150 kilodaltons.

L-Arginine mediates protein-induced insulin secretion (*1, 2*) and potentiates D-glucose–induced insulin release. Furthermore, L-arginine deficiency is associated with insulinopenia and a failure to secrete insulin in response to glucose (*3*). The properties of L-arginine that have been proposed to explain its insulin-releasing effect include nonspecific positive charge and pH effects, stimulation of voltage-sensitive Ca^{2+} channels, and utilization of L-arginine as a substrate for polyamine biosynthesis or transglutaminase (*4*). However, none of these characteristics satisfactorily explains the effect on insulin release. We now show that in three experimental model systems—the anesthetized rat, isolated islets of Langerhans from rat, and the glucose-responsive clonal pancreatic B cell line HIT-T15—the signal transduction pathway involving L-arginine–derived NO (*5*) mediates insulin release from pancreatic B cells.

In HIT-T15 pancreatic B cells, a cell line that retains characteristics of the normal islet insulin secretory response (*6*), L-arginine in the presence of D-glucose induced concomitant release of insulin and NO (Fig. 1). In each case, about 80% of maximal release occurred in the first 10 min. In addition, the median effective concentrations (EC_{50}'s) at which L-arginine potentiated release of insulin and NO were 150 μM and 50 μM, respectively; these values are in the physiological range of plasma L-arginine concentrations (7). L-Arginine (0.3 mM) reduced the EC_{50} for D-glucose to induce release of both NO and insulin (from 15 mM to 5 mM). L-Glucose or D-arginine (up to 30 mM) were without effect (*8*). Tolbutamide, a drug that induces hypoglycemia, also caused release of NO (Fig. 1B) and insulin (*8*) in the presence of D-glucose.

N^G-methyl-L-arginine (MeArg) and N^G-nitro-L-arginine (NO_2Arg), two potent inhibitors of the enzymatic conversion of L-arginine to NO (*9, 10*), inhibited insulin secretion induced by L-arginine in the presence of D-glucose both in vivo, in the anesthetized rat (Fig. 2A), and in vitro, in HIT-T15 cells (Fig. 2B) and isolated islets from rat [maximum inhibition, 57.8 and 45.8% at 100 μM MeArg and NO_2Arg, respectively (*8*)]. Higher concentrations (>300 μM) of either inhibitor were less effective. The observed order of potency (that is, MeArg > NO_2Arg) has not been reported for any other constitutive NO synthase (*9*) and therefore may be relevant for attempts at selective pharmacological intervention in the oxidative L-arginine pathway.

We considered the possibility that endogenous NO and exogenous nitrovasodilators release insulin from pancreatic B cells by stimulating soluble guanylyl cyclase (GC-S) (*11, 12*) and increasing the amount of intracellular guanosine 3′,5′-monophosphate (cGMP), which has been suggested to be a mediator of insulin secretion (*13*). We found that L-arginine (up to 0.3 mM) with D-glucose (10 to 20 mM), in the absence or presence of isobutyl-methylxanthine (IBMX, 1 mM), induced small increases in the amount of cGMP in rat pancreatic islets (59% increase at 3 min) and in HIT-T15 cells (48% increase at 2 min). Direct exposure to the NO generator 3-morpholinosydnonimine (SIN-1, up to 100 μM) also elicited a small elevation in basal cGMP concentrations in HIT-T15 cells (43% increase at 2 min). Supernatant fractions of HIT-T15 cells caused an accumulation of cGMP in rat lung fibroblast (RFL-6) cells rich in GC-S (Fig. 3A), indicating that NO has guanylyl cyclase–activating factor (GAF) bioactivity (*12*). This activity was abolished in the presence of 3 mM EGTA (free Ca^{2+} < 10^{-9} M) or 10 μM hemoglobin or in

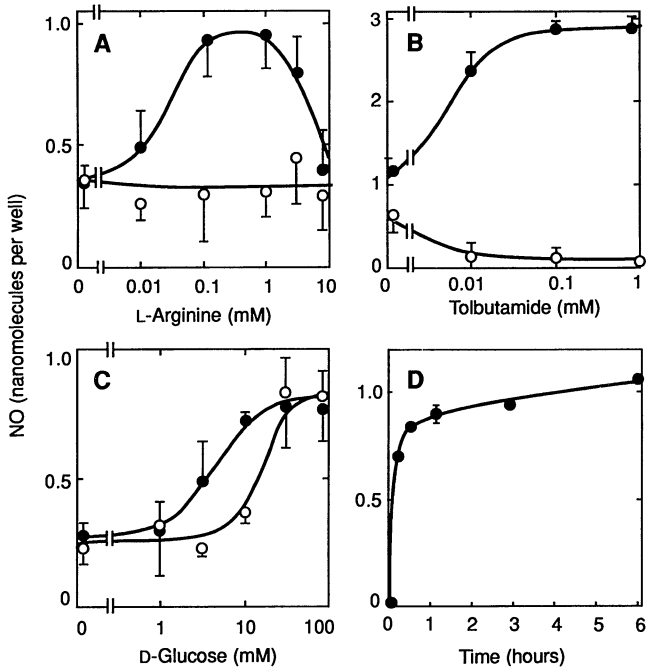

Fig. 1. Release of NO from HIT-T15 clonal pancreatic B cells. Accumulation of NO was measured in the culture medium of cells stimulated for 1 hour with L-arginine in the absence (○) or presence (●) of 10 mM D-glucose (**A**), tolbutamide in the absence (○) or presence (●) of 10 mM D-glucose and 0.3 mM L-arginine (**B**), or D-glucose in the absence (○) or presence (●) of 0.3 mM L-arginine (**C**). (**D**) Release of NO from cells stimulated with 10 mM D-glucose and 0.3 mM L-arginine for various times. HIT-T15 cells (passage 64 to 79) were cultured in RPMI 1640 (Gibco) (*6*) in six-well dishes (3×10^6 cells per well). Cells or islets were equilibrated for 1 hour in glucose-free Hanks buffered salt solution containing bovine serum albumin (3% w/v) (HBSS) before each experiment. After reduction of nitrate to nitrite with nitrate reductase and oxidation of remaining NADPH with lactate dehydrogenase and pyruvate, the amount of accumulated NO (that is, nitrite and nitrate) was measured spectrophotometrically by means of the Griess reaction. Data are averages ± SE of six to eight experiments, each performed in triplicate. NO release correlated with the release of immunoreactive insulin (*8*). Pancreatic islets were isolated as described (*28*) for other experiments.

H. H. H. W. Schmidt, T. D. Warner, F. Murad, Department of Pharmacology, Northwestern University Medical School, Chicago, IL 60611.
K. Ishii, Department of Pharmacology, University of Shizuoka School of Pharmaceutical Sciences, 395 Yada, Shizuoaka-shi, Skizuoka-ken 422, Japan.
H. Sheng and F. Murad, Abbott Laboratories, Abbott Park, IL 60064.

*To whom correspondence should be addressed.

the absence of superoxide dismutase.

The conversion of L-arginine to NO in cell-free preparations of HIT-T15 cells (Fig. 3A) is probably due to the activity of a type I NO synthase, an NO synthase that is soluble and dependent on Ca^{2+}, calmodulin, and NADPH. This hypothesis is supported by our finding that the Ca^{2+} ionophore A23187 (EC_{50}, 80 nM), which is known to induce insulin release (*14*), also induced NO release from HIT-T15 cells (2.89 nmol per well). D-Glucose potentiated the action of A23187 (EC_{50} reduced to 10 nM) but the maximal effects of A23187 and D-glucose were not additive. In addition, insulin secretion is correlated with the intracellular concentration of NADPH (*15*), Ca^{2+} (*14*), and calmodulin (*16*). Direct evidence for the presence of NO synthase in pancreatic B cells was obtained by SDS–polyacrylamide gel electrophoresis (PAGE) and protein immunoblot analysis. Antisera to purified NO synthase from rat cerebellum (type I, 160 kD) (*12, 17*) recognized a 150-kD protein in supernatant fractions of HIT-T15 cells (Fig. 3B) and specifically stained the islets of Langerhans of the rat pancreas (Fig. 4). Islets of Langerhans also contained NADPH diaphorase, an enzyme activity that in brain is identical with NO synthase (*18*). The fact that NADPH diaphorase was detected in the periphery of the islets indicates that pancreatic A_2 cells (cells containing glucagon) also contain NO synthase. Indeed, L-arginine–induced glucagon release has been demonstrated (*19*). The lower inhibitory potency of NO_2Arg relative to MeArg and the lower molecular size of the enzyme on protein immunoblots indicate that NO synthase in B cells is likely to be a different isoform or variant of the type I NO synthase found in brain.

Maximal activation of the oxidative L-arginine signal transduction pathway in some cells and tissues can be limited by the extra-

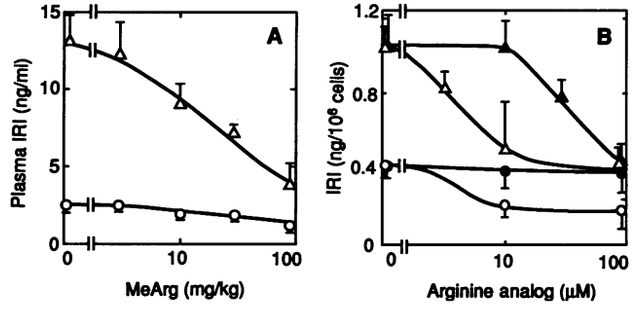

Fig. 2. Effects of NO synthase inhibitors on insulin secretion. (**A**) Effect of N^G-methyl-L-arginine (MeArg) on plasma immunoreactive insulin (IRI) in the anesthetized rat in the absence (○) or presence (△) of D-glucose (750 mg/ml). (**B**) Effect of MeArg (○ and △) or N^G-nitro-L-arginine (● and ▲) on the release of IRI from HIT-T15 cells in the absence (○ and ●) or presence (△ and ▲) of D-glucose (10 mM). Rats (male Wistar, 300 g) were anesthetized (with Inactin; 100 mg/kg, intraperitoneally) and the carotid artery and jugular vein were canulated. Blood (0.5 ml) was collected from the carotid artery immediately before and 5 min after injection of D-glucose (750 mg/kg) via the jugular vein. MeArg was given intravenously as a single injection at the indicated doses immediately after the first sample of blood was collected. Blood samples were centrifuged (4500*g*, 20 min), and the concentration of plasma IRI was determined by radioimmunoassay (ICN). Data are averages ± SE of four to seven experiments. HIT-T15 cells were incubated as described (Fig. 1). Data represent averages ± SE of six to eight experiments, each performed in triplicate.

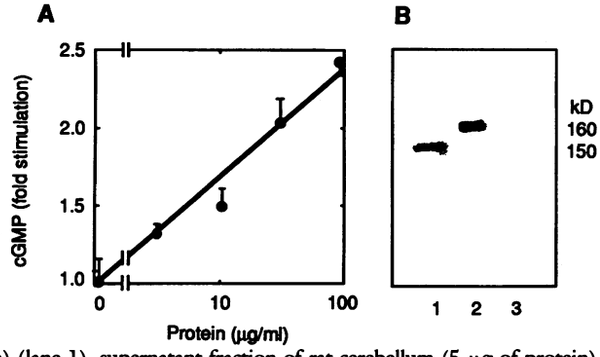

Fig. 3. Detection of NO synthase in HIT-T15 cells. (**A**) NO synthase activity in the supernatant fraction of HIT-T15 cells was assayed by measuring the amount of NO produced, which directly stimulates cGMP levels in RFL-6 cells rich in soluble guanylyl cyclase (*10*). Data are averages ± SE of three experiments, each performed in duplicate. (**B**) Protein immunoblot analysis with antibody to NO synthase–type I (*12*) of crude supernatant fraction of HIT-T15 cells (100 μg of protein) (lane 1), supernatant fraction of rat cerebellum (5 μg of protein) (lane 2), or particulate fraction of HIT-T15 cells (100 μg of protein) (lane 3). HIT-T15 cells (2×10^8; packed cell volume, 0.25 ml) or rat brains (20 g) were homogenized in four volumes of 50 mM tris-HCl (pH 7.45) containing 2-mercaptoethanol (0.1%, v/v), 2 mM EGTA, 1 mM EDTA, and various protease inhibitors (*12*) and centrifuged (105,000*g* for 90 min at 5°C). NO formation in the supernatant fraction of HIT-T15 cells was assayed as described (*10*) in the presence of 100 μM L-arginine, 100 μM NADPH, 1 μM tetrahydrobiopterin, superoxide dismutase (20 U/ml), calmodulin (20 U/ml), and 2 mM Ca^{2+}. Proteins were separated by SDS-PAGE (7.5% gel, w/w) and transferred to nitrocellulose membranes for protein immunoblotting. For immunodetection, all procedures were performed in tris-buffered saline [40 mM tris-HCl (pH 7.55), 0.3 M NaCl] containing Tween 20 (0.3%, v/v). Membranes were subsequently incubated with dried milk (7%, w/v), rabbit polyclonal antiserum (6761-8) to NO synthase–type I (diluted 1:1000) (*12*), and a horseradish peroxidase–conjugate of affinity-purified goat antiserum to rabbit immunoglobulin G (diluted 1:5000) (Sigma). Immune complexes were detected by exposing photographic film to hydrogen peroxide–luminol chemiluminescence (Amersham) and quantitating the density of bands with a laser scanner (AppleScan).

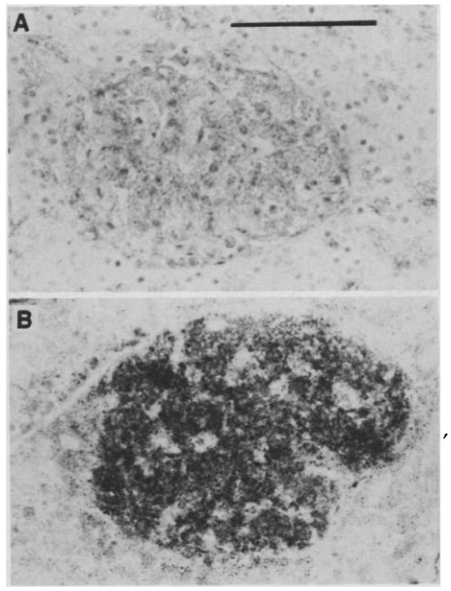

Fig. 4. Detection of NO synthase in islets of Langerhans of rat pancreas. (**A**) Immunohistochemical localization of NO synthase and (**B**) histochemical localization of NADPH diaphorase. Tissues were excised, frozen in hexane–dry ice, and mounted onto microtome chucks with OCT mounting medium. Sections (8 μm) were cut on a Reichert-Jung Frigocut 2800 cryomicrotome, thaw-mounted onto microscope slides, fixed by immersion in acetone at 4°C for 5 min, and air-dried. Slides were stored at 4°C until used. For immunohistochemical staining of NO synthase, slides were incubated in phosphate-buffered saline (PBS) for 5 min, and then with rabbit polyclonal antiserum (6761-8) to NO synthase–type I (diluted 1:100 in PBS containing bovine serum albumin (1%, w/v) for 30 min at 37°C. Slides were then washed twice in PBS (5 min) and incubated in horseradish peroxidase–conjugate of affinity-purified polyclonal goat antiserum to rabbit immunoglobulin G (diluted 1:300). Slides were again washed twice in PBS (5 min) and once with tris-HCl buffer (pH 7.6) (10 min). The peroxidase label was developed with diaminobenzidine dissolved in imidazole buffer (pH 7.6) (6 to 10 min), and the slides were washed in tris-HCl buffer and dehydrated. For histochemical staining of NADPH diaphorase, slides were immersed for 20 to 30 min at 37°C in 50 mM tris-HCl (pH 8.0) containing 1 mM NADPH, 0.5 mM nitroblue tetrazolium, and Triton X-100 (0.2%, v/v). They were then washed briefly in PBS, counterstained with eosin, and dehydrated in a graded series of ethyl alcohol solutions. Slides from both staining procedures were mounted with Permount. The bar indicates 100 μm.

cellular L-arginine concentration (*5, 20*). The concentration of L-arginine required for maximal uptake in endothelial cells is within the physiological range of L-arginine plasma levels (7) [apparent half-maximal saturation constant (K_t) = 0.21 mM; saturation after 15 s]. In contrast, L-arginine uptake in islets is saturated at 15 mM (*21*), a concentration that is about 100 times higher than the normal concentration in plasma. Thus, changes in plasma L-arginine due to diet or other factors would be expected to be a regulator of the oxidative L-arginine pathway in pancreatic B cells but not in endothelial cells.

Exocrine and endocrine stimulus-secretion coupling may represent a physiological function for signal transduction by NO (*22*). The regulation of insulin release by L-arginine has been reported to be deficient in patients with noninsulin-dependent diabetes mellitus (*23*) but not with insulin-dependent diabetes mellitus (*24*). Thus, alterations of the oxidative L-arginine pathway may participate as one of the mechanisms of pathogenesis in diabetes mellitus. Diabetes mellitus may represent only one expression of a putative defect in the oxidative L-arginine pathway. Indeed, diabetes mellitus has been associated with diminution in endothelium-dependent relaxation (*25*) and nonadrenergic, noncholinergic neurotransmission (*26*); both of these processes are known to be mediated by the oxidative L-arginine pathway. These clinical conditions may be explained by altered release of NO or the expression and regulation of different isoforms of NO synthases (*12, 22, 27*).

Note added in proof: L-Arginine–induced increases in cGMP, evidence for a constitutive NO synthase, and inhibition of insulin release by Me Arg in islets of Langerhans from rat and RINm5F insulinoma cells have been reported (*29*).

REFERENCES AND NOTES

1. J. C. Floyd, Jr., S. S. Fajams, J. W. Conn, R. F. Knopf, J. Rull, *J. Clin. Invest.* **45**, 1487 (1966).
2. U. Panten, in *Handbook of Experimental Pharmacology*, A. Hasselbladt and F. von Bruchhausen, Eds. (Springer-Verlag, Berlin, 1975), pp. 115–129.
3. A. L. Mulloy, F. W. Kari, W. J. Visek, *Horm. Metabol. Res.* **14**, 471 (1982).
4. A. Sener, J. Blachier, J. Rasschaert, W. J. Malaisse, *Endocrinology* **127**, 107 (1990); T. Bjaaland and S. L. Howell, *Diabetologica* **32**, 467A (1990).
5. H. H. H. W. Schmidt *et al.*, *Eur. J. Pharmacol.* **154**, 213 (1988); R. M. J. Palmer, D. S. Ashton, S. Moncada, *Nature* **333**, 664 (1988); L. J. Ignarro, G. M. Buga, K. S. Wood, R. E. Byrns, G. Chaudhuri, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 9265 (1987).
6. S. J. Hughes, J. G. Chalk, S. J. H. Ashcroft, *Biochem. J.* **267**, 227 (1990).
7. G. E. Mann, C. J. Sheriff, J. D. Pearson, in *Nitric Oxide from L-Arginine*, S. Moncada and A. E. Higgs, Eds. (Excerpta Medica, Amsterdam, 1990), pp. 331–339.
8. H. H. H. W. Schmidt, T. D. Warner, F. Murad, unpublished data.
9. S. S. Gross *et al.*, *Biochem. Biophys. Res. Commun.* **170**, 96 (1990).
10. K. Ishii, B. Chang, J. F. Kerwin, Jr., Z.-J. Huang, F. Murad, *Eur. J. Pharmacol.* **176**, 219 (1990).
11. H. Kimura, C. K. Mittal, F. Murad, *J. Biol. Chem.* **250**, 8016 (1975).
12. H. H. H. W. Schmidt *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 365 (1991).
13. S. G. Laychock, *Endocrinology* **108**, 1197 (1981).
14. C. B. Wollheim, B. Blondel, P. A. Trueheart, A. E. Renold, G. W. G. Sharp, *J. Biol. Chem.* **250**, 1354 (1975).
15. U. Panten and J. Christians, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **276**, 55 (1973).
16. J. J. Gagliardino, D. E. Harrison, M. R. Christie, E. E. Gagliardino, S. J. H. Ashcroft, *Biochem. J.* **192**, 919 (1980).
17. B. Mayer, M. John, E. Böhme, *FEBS Lett.* **277**, 215 (1990); D. S. Bredt *et al.*, *Nature* **351**, 714 (1991).
18. B. T. Hope, G. J. Michael, K. M. Knigge, S. R. Vincent, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 2811 (1991).
19. T. Bjaaland, P. M. Jones, S. L. Howell, *Biochem. Soc. Transact.* **16**, 1017 (1988).
20. U. Förstermann *et al.*, *Mol. Pharmacol.* **38**, 7 (1990).
21. B. Hellman, J. Sehlin, I. B. Täljedahl, *Endocrinology* **89**, 1432 (1971).
22. S. H. Snyder and D. S. Bredt, *Trends Pharmacol. Sci.* **12**, 125 (1991).
23. W. K. Ward, D. C. Bolgiano, B. McKnight, J. B. Halter, J. Porte, D., *J. Clin. Invest.* **74**, 1318 (1984).
24. O. P. Ganda *et al.*, *Diabetes* **33**, 516 (1984).
25. K. Kamata, N. Miyata, Y. Kasuya, *Br. J. Pharmacol.* **97**, 614 (1989).
26. G. N. Luheshi and M. A. Zar, *ibid.* **101**, 411 (1990).
27. U. Förstermann, J. S. P. Pollock, H. H. H. W. Schmidt, M. Heller, F. Murad, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 1788 (1991).
28. E. J. Verspohl and H. P. T. Ammon, *Naunyn-Schmiedeberg's Arch. Pharmacol.* **339**, 348 (1989).
29. S. G. Laychock, M. E. Modica, C. T. Cavanaugh, *Endocrinology* **129**, 3043 (1991).

30. Appreciation is extended to Z.-J. Huang and T. Gray for expert technical assistance, G. D. Gagne, M. F. Miller, and J. Cerny for preparation of tissue sections, and R. L. Medcalf and H. Majewski for helpful discussions. Support by NIH research grants DK 30787 and HL 28474.

25 September 1991; accepted 18 November 1991

Reversal of Integration and DNA Splicing Mediated by Integrase of Human Immunodeficiency Virus

SAMSON A. CHOW, KAREN A. VINCENT, VIOLA ELLISON, PATRICK O. BROWN*

In retroviral integration, the viral integration protein (integrase) mediates a concerted DNA cleavage-ligation reaction in which the target DNA is cleaved and the resulting 5′ ends of target DNA are joined to the 3′ ends of viral DNA. Through an oligonucleotide substrate that mimics the recombination intermediate formed by this initial cleavage-ligation reaction, the purified integrase of human immunodeficiency virus was shown to promote the same reaction in reverse, a process called disintegration. Analysis of a set of structurally related substrates showed that integrase could promote a range of DNA cleavage-ligation reactions. When the viral DNA component of the disintegration substrate was single-stranded, integrase could mediate a DNA splicing reaction analogous to RNA splicing.

INTEGRATION OF VIRAL DNA INTO THE genome of a new host cell is a critical step in the life cycle of retroviruses. Genetic and biochemical studies of several retroviruses, including human immunodeficiency virus (HIV-1), have shown that integration requires sequences at the ends of the linear viral DNA, and a protein encoded by the viral *pol* gene, the integration protein or integrase (*1, 2*). The viral DNA precursor for the integration reaction is a linear double-stranded molecule that is synthesized by reverse transcription from the viral RNA genome. Two bases from each 3′ end of the linear viral DNA are first removed by integrase such that the viral 3′ ends are recessed by two bases from the 5′ ends and terminate with the dinucleotide CA (*3–8*). A staggered cut is then made in the target DNA, and the resulting overhanging 5′-P ends are covalently joined to the recessed 3′-OH ends of the viral DNA (*3–5, 9*). Target DNA cleavage appears to be coupled to the joining of viral and target DNA because this step does not require an exogenous energy source (*10, 11*). This cleavage-ligation reaction produces a gapped intermediate; integration is completed by a gap repair process that remains to be characterized.

Recent in vitro studies with synthetic oligonucleotides have shown that the processing of the viral DNA ends and strand transfer can be accomplished by integrase alone (*4, 8*). As the cleavage-ligation reaction mediated by integrase is likely to be isoenergetic, we hypothesized that this step would be reversible. In this study, we an-

S. A. Chow and V. Ellison, Department of Pediatrics, Stanford University Medical Center, Stanford, CA 94305.
K. A. Vincent, Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, CA 94305.
P. O. Brown, Departments of Pediatrics and Biochemistry and Howard Hughes Medical Institute, Stanford University Medical Center, Stanford, CA 94305.

*To whom correspondence should be addressed.