15. All NMR experiments were carried out on a 1.7 mM sample of uniformly labeled (>95%) ^{13}C-^{15}N hIL-1β, at pH 5.4 in 150 mM sodium acetate-d_3 buffer dissolved in 90% H_2O–10% D_2O. The sample of hIL-1β was expressed, labeled, and purified as described (*13*). The spectra were recorded on a Bruker AMX600 spectrometer equipped with a triple resonance self-shielded *z* gradient probe. The 2D H_2O NOE-ROE-^1H-^{13}C HSQC experiments were carried out exactly as described in (*17*) with the use of the water flip-back technique to avoid saturating the water (*16*). The NOE spectra were recorded with mixing times of 60, 100, and 200 ms at 35°C and 60 ms at 20°C, whereas the ROE spectra were recorded at mixing times of 30 ms at both 20° and 35°C. In addition, a control H_2O-NOE-^1H-^{13}C HSQC spectrum with a mixing time of 100 ms was recorded with weak presaturation of the water resonance followed by a 200-ms delay before the first selective ^1H pulse (*17*) [S. Grzesiek *et al.*, *J. Am. Chem. Soc.* **116**, 1581 (1994)]. In this control spectrum, all interactions with water were attenuated (about 20-fold), whereas NOEs to protons attached to ^{12}C, which resonate in the vicinity of the water resonance, were essentially unaffected. All the cross-peaks observed in the H_2O-NOE^1-H-^{13}C HSQC difference spectra were suppressed in the control difference spectrum, which indicates that they arise solely from interactions with water (either direct or indirect by means of an exchangeable proton). By recording NOE spectra at several mixing times, as well as ROE spectra, we could ascertain that the cross-peaks attributable to NOEs with water were not the result of spin-diffusion. The spectra were processed with NmrPipe [F. Delaglio *et al.*, in *Proceedings of the 35th Experimental Nuclear Magnetic Resonance Conference* (Asilomar, CA, abstract WP108, 1994), p. 262] and were displayed and analyzed with the programs CAPP and PIPP [D. S. Garrett, R. Powers, A. M. Gronenborn, G. M. Clore, *J. Magn. Reson.* **95**, 214 (1991)]. The spectral assignments were taken from G. M. Clore, A. Bax, P. C. Driscoll, P. T. Wingfield, and A. M. Gronenborn [*Biochemistry* **29**, 8172 (1990)].
16. S. Grzesiek and A. Bax, *J. Biomol. NMR* **3**, 627 (1993); *J. Am. Chem. Soc.* **115**, 12593 (1993).
17. G. M. Clore, A. Bax, J. G. Omichinski, A. M. Gronenborn, *Structure* **2**, 89 (1994).
18. J. Qin, G. M. Clore, A. M. Gronenborn, *ibid.*, p. 503.
19. L. R. Pratt and D. Chandler, *J. Chem. Phys.* **73**, 3430 (1980); G. Ravishanker, M. Mezei, D. L. Beveridge, *Faraday Symp. Chem. Soc.* **17**, 70 (1982); G. I. Makhatadze and P. L. Privalov, *J. Mol. Biol.* **232**, 639 (1993); P. L. Privalov and G. I. Makhatadze, *ibid.*, p. 660; J. Walshaw and J. M. Goodfellow, *ibid.* **231**, 392 (1993).
20. Given that bulk water has a concentration of 55 M, the number of water molecules per cubic angstrom was calculated to be 0.033.
21. F. J. Lovas, *J. Phys. Chem. Ref. Data* **7**, 1445 (1978).
22. The dielectric boundary between the spherical region representing the water molecule and the surrounding medium induces a reaction field E_r at the center of the spherical region. Using boundary conditions of the electrostatic potential, one finds (*29*)

$$E_r = 2(\varepsilon_m - \varepsilon_w)\mu/(2\varepsilon_m + \varepsilon_w)\varepsilon_w a^3$$

where ε_w and ε_m are the dielectric constants of the water molecule and surrounding medium, respectively, and *a* is the radius of a water molecule. The electrostatic interaction energy of the dipole with the surrounding medium is

$$-E_r\mu/2 = -(\varepsilon_m - \varepsilon_w)\mu^2/(2\varepsilon_m + \varepsilon_w)\varepsilon_w a^3$$

(a factor of 332 should be inserted if μ is in units of eÅ, *a* in units of Å, and the energy in units of kcal mol^{-1}). The dielectric constant ε_w of the spherical region representing the water molecule is due to electronic polarization (*29*) and should be small. We thus chose $\varepsilon_w = 2$. The change in the interaction free energy of a water molecule with surrounding water molecules from the vapor phase to the liquid phase has been estimated from the vapor pressure and density of water and is found to be −6.3 kcal mol^{-1} at 25°C [A. Ben-Naim and Y. Marcus, *J. Chem. Phys.* **81**, 2016 (1984)]. This is very close to the value (−7.1 kcal mol^{-1}) predicted by our simple model for the change in electrostatic interaction energy. The model gives +2.4 and −4.7 kcal mol^{-1}, respectively, for the electrostatic interaction energy in the vapor phase ($\varepsilon_m = 1$) and in the liquid phase ($\varepsilon_m = 78.5$). A small (that is, <1 kcal mol^{-1}) nonelectrostatic component that disfavors the liquid phase would further improve the agreement between the model prediction and the estimate from experimental data.
23. H.-X. Zhou, *Biophys. J.* **65**, 955 (1993).
24. We obtained the electrostatic potential by solving discrete versions of the integral equations, describing the electrostatic potential and its normal derivative on the protein surface as previously done (*23*). Human IL-1β has an overall charge of −*e*.
25. M. F. Perutz, G. Fermi, D. Abraham, C. Poyart, E. Bursaux, *J. Am. Chem. Soc.* **108**, 1064 (1986); M. Levitt and M. F. Perutz, *J. Mol. Biol.* **201**, 751 (1988).
26. C. Chothia, *Nature* **248**, 338 (1974).
27. A. Ben-Naim, *Solvation Thermodynamics* (Plenum, New York, 1987).
28. D. H. Ohlendorf, A. C. Terharne, P. C. Weber, J. J. Wendoloski, F. R. Salemme, Brookhaven Protein Data Bank, accession code 8I1B (1991); X. Zhu *et al.*, *Science* **251**, 90 (1991); Z. Zhang, L. S. Cousens, P. J. Barr, S. R. Sprang, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 3446 (1991); S. Onesti, P. Brick, D. M. Blow, *J. Mol. Biol.* **217**, 153 (1991).
29. T.-W. Lee and R. Zwanzig, *J. Chem. Phys.* **52**, 6353 (1970).
30. A. J. Nicholls, K. Sharp, B. Honig, *Proteins* **11**, 281 (1991).
31. We thank D. Garrett and F. Delaglio for software development; R. Tschudin for developing and building the pulsed field gradient accessory; and A. Bax, K. Frank, S. Grzesiek, A. Karplus, J. Kuszewski, P. Lodi, J. Qin, A. Szabo, and A. Wang for numerous helpful discussions. R.T.C. acknowledges a Leukemia Society of America postdoctoral fellowship. Supported by the AIDS Targeted Anti-Viral Program of the Office of the Director of the National Institutes of Health (G.M.C. and A.M.G.).

7 October 1994; accepted 12 January 1995

DNA Topoisomerase and Recombinase Activities in Nae I Restriction Endonuclease

Kiwon Jo and Michael D. Topal*

Nae I endonuclease must bind to two DNA sequences for cleavage. Examination of the amino acid sequence of Nae I uncovered similarity to the active site of human DNA ligase I, except for leucine 43 in Nae I instead of the lysine essential for ligase activity. Changing leucine 43 to lysine 43 (L43K) changed Nae I activity: Nae I–L43K relaxed supercoiled DNA to yield DNA topoisomers and recombined DNA to give dimeric molecules. Interruption of the reactions of Nae I and Nae I–L43K with DNA demonstrated transient protein-DNA covalent complexes. These findings imply coupled endonuclease and ligase domains and link Nae I endonuclease to the topoisomerase and recombinase protein families.

Topoisomerases, recombinases, and endonucleases share the ability to cleave DNA but do not have sequence homology. Topoisomerases catalyze DNA relaxation by cleavage, strand passage, and reunion; recombinases catalyze DNA rearrangements by concerted cleavage and exchange of DNA ends; and endonucleases catalyze cleavage of single- and double-stranded DNA. These enzymes are ubiquitous and essential for replication, transcription, recombination, and repair of DNA (*1*). Although there has been speculation on their evolution, no relation among these enzymes has been found. Endonucleases include as a special class the restriction enzymes, which in bacteria protect against foreign DNA. The restriction enzymes, along with the site-specific recombinases, offer examples of sequence-specific DNA cleavage. Topoisomerases generally demonstrate sequence preferences but are not sequence-specific.

The placing of restriction enzymes in a separate category from the topoisomerases and recombinases has been called into question by recent discoveries with the type IIe restriction enzymes. It is now clear that the type IIe enzymes require the recognition of a second DNA (effector) sequence to cleave DNA; moreover, there is homology between the type IIe enzyme Eco RII and the integrase family of proteins (*2*). These findings indicate complexities in the type IIe enzymes beyond that needed for restriction. The type IIe enzymes include Nae I (*3*), Nar I, Bsp MI, Hpa II, Sac II (*4*), Eco RII (*5*), Atu BI, Cfr 9I, Sau BMKI, Eco 57I, and Ksp 632I (*6*), which represent a wide variety of bacterial species.

Nae I is a prototypical type IIe enzyme, a 70-kD dimeric protein (*7*) with two DNA binding sites, as indicated by its sigmoidal dependence of cleavage velocity on the concentration of recognition sequence (*8*). The two DNA binding sites of Nae I are nonidentical: one site prefers to bind to GCCGGC with AT-rich flanking sequences, whereas the other prefers to bind to GCCGGC with GC-rich flanking sequences (*8*). Whether the binding differences preexist or are induced upon occupation of one DNA binding site is unclear. Because Nae I must bind two DNA recognition sequences, a DNA substrate with a single Nae I recognition sequence can resist cleavage (*3*). This resistance can be overcome by the introduction of another DNA recogni-

Lineberger Comprehensive Cancer Center, Department of Biochemistry and Biophysics, and Department of Pathology, University of North Carolina Medical School, Chapel Hill, NC 27599, USA.

*To whom correspondence should be addressed.

tion sequence, either on the same molecule (in cis) or in trans by addition of exogenous DNA fragments with affinity for the second DNA binding site on the enzyme (*3*, *7*, *8*).

Nae I also induces loops in DNA, with the enzyme binding at the base of the loops (*9*). The ability of Nae I to juxtapose and cleave distant Nae I recognition sequences in DNA is reminiscent of the recombinases, transposases, and type II topoisomerases. In fact, restriction enzymes appear to function at some level in vivo to produce DNA with recombinogenic ends (*10*, *11*). The introduction of restriction enzymes into yeast (*12*) and mammalian cells (*13*) induces DNA rearrangements. These rearrangements presumably arise through random ligation of DNA fragments made recombinogenic by restriction endonuclease cleavage (*11*). Thus, the presence of endonuclease and ligase activities that function together in the same polypeptide might together make a form of recombinase.

In a computer search with a basic local alignment search tool (*14*) (BLAST network service at the National Center for Biotechnology Information), no similarities were detected between the Nae I amino acid sequence and sequences of the recombinase, transposase, and topoisomerase families. Comparison of the amino acid sequence of Nae I with that of the DNA ligases, another ubiquitous family of proteins used to seal DNA breaks (*1*), disclosed a 10–amino acid region [near the NH_2-terminus of the putative Nae I active site (*15*)] that matched the consensus for the active site of DNA ligase I (Fig. 1). The better matches of Nae I are with the active sites of the eukaryotic rather than the prokaryotic DNA ligases; the best match is with that of human DNA ligase I. The Nae I sequence differs, however, from the human ligase active site in one important respect: the lysine (K) that forms the adenylated intermediate essential for catalysis by the DNA ligase active site is not present in Nae I. Instead, there is a leucine (L43) at this position.

Nae I	39	T L D Q **L** Y D G Q R	48
Human	564	T C E Y **K** Y D G Q R	573
S. pombe	412	T C E Y **K** Y D G E R	421
S. cerevisiae	416	T S E Y **K** Y D G E R	425
Vaccinia	227	F A E V **K** Y D G E R	236
T7	30	I A E I **K** Y D G V R	39
T3	30	I A D C **K** Y D G V R	39
T4	156	F A Q L **K** A D G A R	165
E. coli	111	C C E L **K** L D G L A	120
T. thermophilus	114	T V E H **K** V D G L S	123

Fig. 1. Comparison of an NH_2-terminal region of Nae I with the active-site regions for enzyme-adenylate formation in DNA ligases. The sequences are taken from a review (*17*). The reactive lysine residue is shown in bold. Abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Site-directed mutagenesis of L43 to K43 radically altered Nae I activity. Incubation of pBR322 DNA, which contains four Nae I recognition sites, with reaction-limiting amounts of wild-type Nae I resulted in a partial digest (Fig. 2A, lanes 1 and 2). Incubation of pBR322 DNA with Nae I–L43K resulted in relaxation of the supercoiled DNA in a stepwise manner to yield covalently closed DNA topoisomers (Fig. 2A, lane 4). The bimodal distribution of superhelical and relaxed DNA molecules implies a predominantly processive mode of action under these reaction conditions (*16*), meaning several enzyme cycles before dissociation. The relaxation by Nae I–L43K was identical to that caused by commercial topoisomerase (Fig. 2A, lane 3). After the reaction had gone to completion, half of the supercoiled DNA substrate had been converted by Nae I–L43K to relaxed covalently closed DNA; the other half was nicked (Fig. 2B, lane 3). Thus, this single amino acid change gave Nae I classic topoisomerase activity, which requires DNA cleavage, strand passage, and religation.

The apparent activities of the endonuclease and ligase domains of Nae I–L43K were observed by the effect of Nae I–L43K on covalently closed circular DNA that was completely relaxed. Use of completely relaxed DNA eliminates topoisomers as possible substrates. We prepared such DNA by cleaving and religating pBR322 DNA at the unique Hind III site. Nae I–L43K cleaved this DNA substrate to convert the closed-circle to full-length linear products and used both the cleavage and ligation functions to produce species with a greater molecular weight (Fig. 2C). The products from this reaction, and from reactions in which Nae I–L43K was allowed to completely relax supercoiled pBR322 DNA, were found by electron microscopy to contain double-length circular and linear molecules as well as the expected unit-length circles and cleaved products in proportions consistent with the proportions of the major species resolved by gel electrophoresis

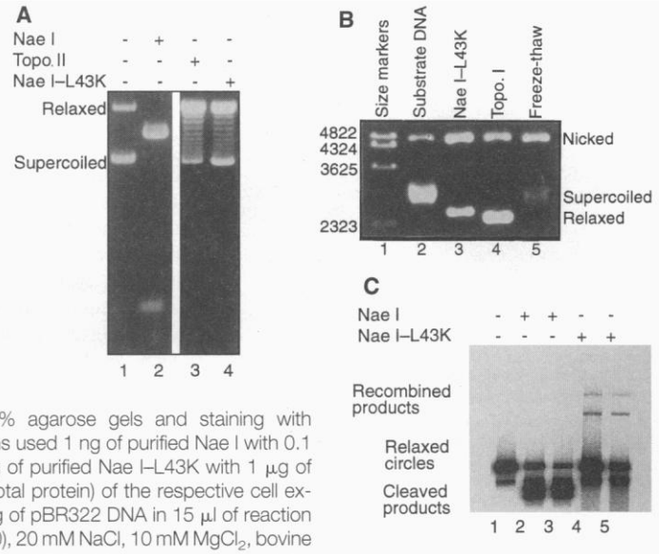

Fig. 2. Effect of the L43K mutation on the activity of Nae I. (**A**) The products of incubating wild-type Nae I and Nae I–L43K with pBR322 DNA. Lane 1, pBR322 DNA only; lane 2, pBR322 DNA incubated with wild-type Nae I; lane 3, incubated with commercial *Drosophila* topoisomerase II (Topo. II) (U.S. Biochemical); lane 4, incubated with purified Nae I–L43K. Products were resolved by electrophoresis through 1% agarose gels and staining with ethidium bromide. Reactions used 1 ng of purified Nae I with 0.1 μg of pBR322 DNA, 30 ng of purified Nae I–L43K with 1 μg of pBR322 DNA, and 1 μg (total protein) of the respective cell extracts (not shown) and 1 μg of pBR322 DNA in 15 μl of reaction buffer [10 mM tris-Cl (pH 8.0), 20 mM NaCl, 10 mM $MgCl_2$, bovine serum albumin (0.1 mg/ml), and 5.0 mM β-mercaptoethanol] for 50 min at 37°C. (**B**) Complete relaxation of pBR322 DNA by Nae I–L43K produces closed circular DNA. The reaction in (A) was taken to completion and the products analyzed on a 1% agarose gel containing ethidium bromide (0.5 μg/ml). Lane 1, size (bp) markers (Bst EII digest of λ DNA); lane 2, pBR322 DNA without enzyme; lane 3, pBR322 DNA completely relaxed with Nae I–L43K; lane 4, pBR322 DNA completely relaxed with calf thymus topo. I (BRL); lane 5, pBR322 nicked by repeated cycles of freezing and thawing. The differences between the mobilities of the closed circular products of Nae I–L43K and topo. I is probably caused by a different extent of relaxation. The relative amounts of nicked and relaxed DNA were determined by densitometry after corrections for the amount of nicked DNA in the substrate and for differences in ethidium bromide staining. Amounts of relaxed DNA were 50 and 75% for Nae I–L43K and topo. I, respectively. (**C**) Effect of wild-type Nae I and Nae I–L43K on relaxed, covalently closed circular pBR322 DNA. Products were resolved by electrophoresis through 1% agarose gels and detected with the use of a Molecular Dynamics phosphorimager. Plasmid pBR322 DNA was cleaved with Hind III, radiolabeled, and religated, and the closed circular form was gel-purified. This DNA (approximately 0.2 μg) (lane 1) was used as substrate for purified wild-type Nae I (5 ng) (lane 2, products after treatment with proteinase K; lane 3, no proteinase K) and purified Nae I–L43K (500 ng) (lane 4, products plus proteinase K; lane 5, no proteinase K) in 15 μl of reaction buffer. Reactions containing wild-type Nae I were incubated for 10 min at 37°C; reactions containing Nae I–L43K were incubated for 1 hour at 37°C.

(Fig. 3). These results demonstrate that Nae I–L43K can recombine DNA as well as relax supercoiled DNA to give topoisomers.

Cell extracts without the overexpressed Nae I–L43K mutant or that overexpress Nae I–E70K (where E is Glu), a variant that binds to but cannot cleave DNA (*15*), lacked the topoisomerase activity. Thus, the enriched topoisomerase activity is specific to the L43K variant. The Nae I–L43K activity was purified to apparent homogeneity. All ligases require adenosine triphosphate (ATP) or nicotinamide adenine dinucleotide (NAD) for activity (*17*). Purified Nae I–L43K, however, processively reduced the linking number of pBR322 DNA (Fig. 2A) and produced higher molecular weight products (Fig. 2C) without an outside energy source; ATP, guanosine triphosphate, and NAD had no measurable effect on Nae I–L43K activity. Moreover, under conditions that produced this intermediate when we used both bacteriophage T4 DNA ligase with labeled ATP and *Escherichia coli* DNA ligase with labeled NAD, we did not detect an adenylated intermediate when we used Nae I–L43K with either radiolabeled ATP or NAD. Thus, the ligase motif in Nae I does not act as a classic ATP or NAD ligase.

This independence from an outside energy source implied that either Nae I–L43K alone or both Nae I–L43K and wild-type Nae I (which performs only the cleavage step) form activated covalent protein-DNA intermediates. An activated intermediate was implicated by the DNA relaxation activity of Nae I–L43K because energy is required for ligation, but the cleavage and ligation steps in DNA relaxation are interrupted by a DNA strand-passage step. Such covalent intermediates are common energy sources for strand breakage and reunion among the topoisomerases (*18*) and recombinases (*19*). To observe this intermediate for Nae I and Nae I–L43K, we used heat to interrupt the reaction with radiolabeled DNA substrate. Extensive nuclease treatment of the reaction mixture transferred the radioactive label from the DNA substrate to protein: the nuclease-treated, radiolabeled Nae I–DNA complex survived boiling in SDS and gave a denatured protein band by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) with an apparent molecular weight consistent with that of Nae I (Fig. 4). Nae I–L43K produced a similar result, but far less labeled protein was produced. The more transient nature of the intermediate in the L43K mutant compared to that in wild-type Nae I is consistent with a concerted displacement of the protein during religation.

The mechanism of displacement of the activated Nae I–DNA intermediate appears to be analogous to that found in DNA ligases. The covalent intermediate formed between wild-type Nae I and pBR322 DNA indicates that the activated intermediate is formed during the cleavage step, independent of the L43K mutation. Thus, the unadenylated ligase motif of Nae I–L43K may use the activated DNA-protein intermediate formed during the cleavage step for resealing DNA breaks, analogous to the use by unadenylated DNA ligase of an activated DNA–adenosine monophosphate intermediate for sealing DNA breaks (*1*).

Nae I is highly specific for its recognition sequence: it binds and cleaves 10^{11}-fold more efficiently at its cognate sequence than at noncognate recognition sequences (*20*). To test for sequence discrimination, we used different DNAs as substrates for Nae I–L43K. Plasmid pUC18 lacks a Nae I recognition sequence; M13 double-stranded DNA has one such sequence that is resistant to cleavage by Nae I because of its poor affinity for the Nae I effector-binding site (*3*, *8*). Neither of these DNAs was a substrate for Nae I–L43K. Plasmid pBR322 has four Nae I recognition sequences and is a good substrate for wild-type Nae I; this plasmid was also a good substrate for Nae I–L43K (Fig. 5). These results imply that the topoisomerase activity of Nae I–L43K is sequence-dependent and demonstrate the relation of Nae I–L43K topoisomerase activity to Nae I protein.

The lack of homology between Nae I–L43K and either the topoisomerases or recombinases indicates that Nae I–L43K is a new protein structure for studies of topoisomerase and recombinase mechanisms. The discovery of topoisomerase and recombinase activities in Nae I and a covalent Nae I–DNA intermediate provides direct evidence for an evolutionary link between the type IIe restriction enzymes and the recombinase protein families. In addition, recombinases are proving to be useful reagents for the directed modification of

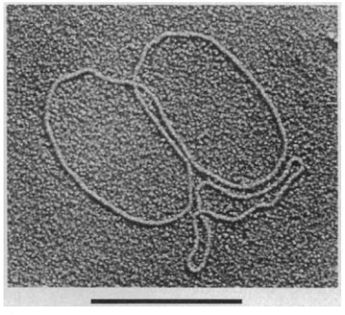

Fig. 3. Electron microscope visualization of a pBR322 dimer formed by the action of Nae I–L43K. Monomer pBR322 DNA circles (smaller circle) were incubated with Nae I as described in the text. Reaction products were treated with SDS to remove protein and purified by gel permeation chromatography. DNA samples were prepared for electron microscopy with the use of the denatured protein monolayer method (*22*). Molecular lengths were determined by measurements taken directly from micrographs.The circle crossing at its center is a dimer. Bar equals 0.5 μm.

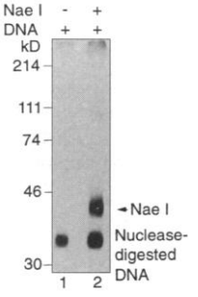

Fig. 4. Covalent complex formation between Nae I and pBR322 DNA. Purified Nae I (0.47 μM) was incubated with 300 ng of pBR322 DNA, which had been uniformly labeled by nick translation, in 15 μl of reaction buffer as described in Fig. 2. The reaction was stopped after 30 min at 37°C by heating to 70°C for 10 min. The reaction products were digested with exonuclease III and deoxyribonuclease I overnight, then SDS was added to the digest to 2% (w/v) and β-mercaptoethanol to 5% (v/v), and the mixture was boiled for 3 min. The reaction products were then resolved by SDS-PAGE (10% acrylamide gel–4% spacer gel and 0.4% SDS) and visualized by autoradiography. Lane 1, no Nae I control; lane 2, result of incubating Nae I with labeled substrate as described. In addition, Nae I protein was denatured by boiling in SDS, electrophoresed, and located by Coomassie blue staining; its mobility in the gel is indicated. Prestained molecular weight markers (carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, and myosin heavy chain) were also run, and apparent molecular weights, as reported by the manufacturer (BRL, Gaithersburg, Maryland), are shown. Similar results to those in lane 2 were obtained with the use of Nae I–L43K in place of Nae I, but smaller amounts of labeled protein were obtained.

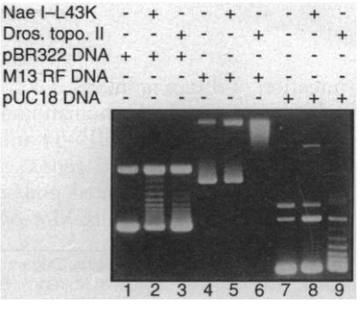

Fig. 5. Reaction of Nae I–L43K with pBR322 (four Nae I recognition sequences), M13 double-stranded DNA (one recognition sequence), and pUC (no recognition sequences). Purified Nae I–L43K (30 ng) (shown) and cell extract (1 μg of total protein) (not shown) were incubated with pBR322 DNA (1.0 μg) (lanes 1 to 3); M13mp18 DNA (0.5 μg) (lanes 4 to 6); and pUC DNA (0.75 μg) (lanes 7 to 9) under reaction conditions indicated in Fig. 2. Products from all reactions were resolved by gel electrophoresis (1% agarose) and visualized by staining with ethidium bromide. Dros. topo. II, *Drosophila* topoisomerase II.

higher eukaryotic organisms (*21*). Nae I–L43K opens the possibility of engineering proteins that use similar combinations of sequence-specific endonuclease and ligase domains as valuable in vivo reagents for genomic manipulation at sequence-specific targets.

REFERENCES AND NOTES

1. A. Kornberg and T. A. Baker, *DNA Replication* (Freeman, New York, ed. 2, 1991).
2. M. D. Topal and M. Conrad, *Nucleic Acids Res.* **21**, 2599 (1993).
3. M. Conrad and M. D. Topal, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9707 (1989).
4. A. R. Oller *et al.*, *Biochemistry* **30**, 2543 (1991).
5. D. H. Krüger, G. J. Barcak, M. Reuter, H. O. Smith, *Nucleic Acids Res.* **16**, 3997 (1988); S. Gabbara and A. S. Bhagwat, *J. Biol. Chem.* **267**, 18623 (1992).
6. M. Reuter *et al.*, *Anal. Biochem.* **209**, 232 (1993).
7. B. K. Baxter and M. D. Topal, *Biochemistry* **32**, 8291 (1993).
8. C. C. Yang and M. D. Topal, *ibid.* **31**, 9657 (1992).
9. M. D. Topal *et al.*, *ibid.* **30**, 2006 (1991).
10. R. J. Roberts, *Crit. Rev. Biochem.* **4**, 123 (1976); W. Arber, *J. Struct. Biol.* **104**, 107 (1990).
11. S. Chang and S. N. Cohen, *Proc. Natl. Acad. Sci. U.S.A.* **74**, 4811 (1977).
12. R. H. Schiestl and T. D. Petes, *ibid.* **88**, 7585 (1991).
13. E. Abella-Columna *et al.*, *Environ. Mol. Mutagen.* **22**, 26 (1993).
14. S. F. Altschul *et al.*, *J. Mol. Biol.* **215**, 403 (1990).
15. J. Holtz and M. D. Topal, *J. Biol. Chem.* **269**, 27286 (1994).
16. H.-P. Vosberg, *Curr. Top. Microbiol. Immun.* **114**, 19 (1985).
17. T. Lindahl and D. E. Barnes, *Annu. Rev. Biochem.* **61**, 251 (1992).
18. J. C. Wang, *ibid.* **54**, 665 (1985).
19. N. L. Craig, *Annu. Rev. Genet.* **22**, 77 (1988).
20. C. C. Yang, B. K. Baxter, M. D. Topal, *Biochemistry* **33**, 14918 (1994).
21. B. Sauer and N. Henderson, *New Biol.* **2**, 441 (1990); S. O'Gorman, D. T. Fox, G. M. Wahl, *Science* **251**, 1351 (1991); K. G. Golic, *ibid.* **252**, 958 (1991); J. G. Pichel *et al.*, *Oncogene* **8**, 3333 (1993).
22. R. Thresher and J. Griffith, *Methods Enzymol.* **211**, 481 (1992).

23. We thank T. Maness for expert technical assistance and the Lineberger Comprehensive Cancer Center for financial support. Electron Microscopy for Fig. 3 was courtesy of K. Park, University of North Carolina.

12 September 1994; accepted 30 November 1994

Pathogenicity of Live, Attenuated SIV After Mucosal Infection of Neonatal Macaques

Timothy W. Baba, Yong Seok Jeong, Dominique Penninck, Rod Bronson, Michael F. Greene, Ruth M. Ruprecht*

Adult macaques do not develop disease after infection with a *nef* deletion mutant of the simian immunodeficiency virus (SIV) and are protected against challenge with pathogenic virus. This finding led to the proposal to use *nef*-deleted viruses as live, attenuated vaccines to prevent human acquired immunodeficiency syndrome (AIDS). In contrast, neonatal macaques developed persistently high levels of viremia after oral exposure to an SIV *nef*, *vpr*, and negative regulatory element (NRE) deletion mutant. Severe hemolytic anemia, thrombocytopenia, and CD4$^+$ T cell depletion were observed, indicating that neither *nef* nor *vpr* determine pathogenicity in neonates. Because such constructs have retained their pathogenic potential, they should not be used as candidate live, attenuated virus vaccines against human AIDS.

SIV infection of rhesus monkeys is considered to be the best model for human immunodeficiency virus–type 1 (HIV-1) infection of humans (*1*). The viral genomes of HIV-1 and SIV are closely related, and both viruses infect similar target cells. Moreover, SIV disease in rhesus macaques is comparable to human AIDS; SIV infection of adult macaques results in high levels of virus replication, CD4$^+$ T cell depletion, and immunosuppression (*2*). In contrast, a molecular construct of SIV with a deletion in the auxiliary *nef* gene replicated poorly in adult macaques after intravenous injection (*3*). Although these animals were persistently infected with low amounts of virus, they maintained normal CD4$^+$ T cell counts. During a follow-up of more than 3 years, no signs of immunodeficiency developed. When challenged with pathogenic SIV, these animals were protected from disease (*4*). On the basis of these data, *nef*-deleted mutants have been proposed as live, attenuated virus vaccines to protect humans against HIV-1 (*4*, *5*). Vaccine studies with *nef*-deleted HIV-1 viral constructs are ongoing in chimpanzees (*6*).

T. W. Baba, Division of Newborn Medicine, Department of Pediatrics, Tufts University School of Medicine, Boston, MA 02111, and Laboratory of Viral Pathogenesis, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, MA 02115, USA.
Y. S. Jeong and R. M. Ruprecht, Laboratory of Viral Pathogenesis, Dana-Farber Cancer Institute, and Department of Medicine, Harvard Medical School, Boston, MA 02115, USA.
D. Penninck, Department of Radiology, Tufts University School of Veterinary Medicine, Grafton, MA 01536, USA.
R. Bronson, Department of Veterinary Pathology, Tufts University School of Veterinary Medicine, Boston, MA 02111, USA.
M. F. Greene, Department of Obstetrics and Gynecology, Massachusetts General Hospital, Boston, MA 02114, and Harvard Medical School, Boston, MA 02115, USA.

*To whom correspondence should be addressed.

The data generated from adult macaques infected with the *nef*-deleted SIV also led to the hypothesis that *nef* is a major determinant of pathogenicity in vivo for immunosuppressive lentiviruses (*3*). The *nef* gene product, a myristylated phosphoprotein of 34 kD apparent molecular size, down-regulates CD4 surface antigen expression but is not required for virus replication in vitro in T cell lines (*7*). Other Nef functions are more controversial; various effects on transcription from the HIV-1 long terminal repeat (LTR) have been described (*8*). According to more recent reports (*9*), Nef facilitates virus replication in unstimulated peripheral blood mononuclear cells (PBMCs).

Studying the pathogenicity and prevention of perinatal retrovirus transmission is important, given the increasing numbers of HIV-1–infected children (*10*). It appears that some newborns become infected after mucosal exposure to infectious maternal blood or secretions (*11*). We have developed a primate model of neonatal mucosal infection (*12*). Rhesus monkey (*Macaca mulatta*) neonates were exposed orally to cell-free SIV immediately after delivery. All exposed neonates became infected; high amounts of virus were seen in all animals, and one-half died of AIDS within 6 months.

As a potential live, attenuated vaccine virus, we evaluated a mutant of SIV deleted in *nef*, *vpr*, and NRE, termed SIVΔ3 (*13*). Like *nef*, *vpr* is an accessory gene that is believed to increase virus replication in cultured T cells and monocytes and macrophages (*14*). Vpr is virion-associated and has been implicated in achieving high amounts of virus replication and disease development in vivo (*15*). NRE, a negative regulatory element located in the LTR (*16*), had been removed to ensure adequate viral replication. The resulting triply deleted SIV mutant replicates well in cultured cells, including macaque PBMCs. In adult macaques injected intravenously with an amount of virus containing 5 ng of p27 [corresponding to 2000 50% tissue culture infectious doses ($TCID_{50}$)], virus could be isolated only transiently, but the animals remained seropositive and polymerase chain reaction (PCR)–positive. When challenged intravenously with 10 animal infectious doses of pathogenic SIV_{mac251}, protection was seen (*13*).

Three rhesus monkey neonates were given cell-free SIVΔ3 orally within 1 hour after cesarean section delivery (*17*). The oral SIVΔ3 dose given was 1.6 μg of p27, corresponding to 5.3×10^5 $TCID_{50}$. This dose was ~300 times the dose given intravenously to adult macaques as vaccine previously (*13*); it was chosen because mucosal infection in general requires a higher virus inoculum, as compared with intravenous inoculation (*18*). The selection of the dose