Supporting Information

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SI Materials and Methods

Apoptosis Assays. CHO cells were transfected with the indicated plasmids. The cells were incubated with staurosporine (0.5 μg/mL) for 5 h. The cells were fixed with 3% (wt/vol) paraformaldehyde for 20 min, permeabilized with ice-cold methanol for 30 s, quenched with 50 mM NH_4Cl for 15 min, and labeled with HA-specific antibody (Convance Research Products) and fluorescein-labeled secondary antibody (Molecular Probes) or left unlabeled. Cells were mounted using ProLong Gold with DAPI (Invitrogen) to visualize the nucleus. The nuclear morphology of transfected cells was scored using a Nikon TE-200 inverted microscope.

CHO FcR cells were incubated with anti-*Legionella pneumophila* antibody 30 min before infection with wild-type *L. pneumophila* or with the defect in organelle trafficking (*dotA*) mutant expressing the indicated plasmids at a multiplicity of infection (MOI) of 30 for 1 h (1). The cells were washed with PBS and treated with staurosporine (0.4 μg/mL) for 5 h. *L. pneumophila* were detected using an Alexa Fluor 594 secondary antibody (Molecular Probes). Nuclear DNA fragmentation was assayed by TUNEL with the in situ cell death detection kit (Roche). The nuclei were analyzed as described above.

After being isolated using CD11c magnetic beads (2), dendritic cells (DCs) were infected with *L. pneumophila* at an MOI of 25 and assayed for nuclear DNA fragmentation by TUNEL with the in situ cell death detection kit (Roche). Samples then were analyzed by fluorescence microscopy. All data points represent the average number of TUNEL-positive cells ± SD obtained from three independent coverslips.

Coimmunoprecipitation. HEK 293 cells were transfected with the indicated plasmids. Cells were lysed in lysis buffer [20 mM Hepes (pH 7.5), 200 mM NaCl, 1 mM EDTA, 0.1% (vol/vol) Nonidet P-40, 10% (vol/vol) Glycerol, 1× protease inhibitor, 1 mM DTT] for 30 min on ice and centrifuged for 10 min, 16,000 × *g*. at 4 °C. The supernatants were incubated with anti-GFP rabbit antiserum (Invitrogen) for 3 h at 4 °C. Complexes were precipitated using protein A Sepharose beads (40 min at 4 °C). The beads were washed three times with buffer [20 mM Hepes (pH 7.5), 200 mM NaCl, 1 mM EDTA, 0.1% (vol/vol) Nonidet P-40]. Precipitated proteins were analyzed by immunoblotting.

Immunoblotting. Proteins were separated by SDS/PAGE and transferred to PVDF membrane (Millipore). The membranes were probed with antibodies specific for GFP (Roche), HA (Convance Research Products), and gC1qR (p32) (Santa Cruz). Proteins were visualized using HRP-conjugated secondary antibodies (Zymed) and a chemiluminescence detection system (Perkin-Elmer).

Protein Purification. *Escherichia coli* BL21 (DE3) cells transformed with plasmids producing either GST or GST- ankyrinG (AnkG) were grown to an OD = 0.4. Isopropyl β-D-1-thiogalactopyranoside was added, and samples were incubated for 4 h at 30 °C. Cells were resuspended in PBS containing protease inhibitor. After disruption by French press, the lysates were clarified by centrifugation at 15,000 × *g* for 30 min. Supernatants were loaded onto glutathione–Sepharose columns (GE Healthcare). Bound proteins were eluted with 10 mM glutathione in PBS. Proteins were dialyzed and coupled to Affi-Gel 15 (BioRad) according to the manufacturer's protocol.

CHO cells were resuspended in lysis buffer [0.5% (vol/vol) Triton X-100, 50 mM Tris (pH 7.5), 100 mM NaCl] for 1 h on ice. After centrifugation (20.000 × *g* for 20 min at 4 °C and 100,000 × *g* for 45 min at 4 °C), the supernatant was incubated with GST or GST-AnkG coupled to Affi-Gel for 3–4 h at 4 °C). Beads were washed with lysis buffer and TBS [10 mM Tris (pH 7.5), 150 mM NaCl]. Bound proteins were eluted in 100 mM glycine (pH 2.4) and 150 mM NaCl and neutralized with 1 M Tris (pH 8.0). Eluted proteins were precipitated using trichloroacetic acid and separated by SDS/PAGE. The gel was stained with silver, and unique bands from the GST-AnkG column were excised and analyzed by MALDI-TOF (Protana Analytical Services).

siRNA Knock Down. The Dharmacon protocol for siRNA transfection of HeLa cells was used. Three days after the cells were transfected with 10 nM p32 siRNA, the cells were washed with PBS and exposed to UV light (600 J/m^2) in a transilluminator box (Stratagene). After fresh medium was added, cells were incubated for 5 h. The cells were fixed, permeabilized, quenched, and mounted as described above. The nuclear morphology of cells was scored as described above.

siRNA knock-down in mouse bone marrow DCs was performed as described previously with some alterations (3). Briefly, 8 d after DC differentiation, 6 μg of siRNA single oligonucleotide (A, B, C, D) or 6 μg of a pool of four siRNA oligonucleotides directed against mouse p32 were introduced into the cells by electroporation. DCs electroporated with no siRNA were used as control (mock treatment). The efficiency of knock-down was confirmed 48 h post electroporation by immunoblotting using a p32-specific antibody from Abcam. DCs then were sorted using CD11c magnetic beads (Miltenyi Biotec) and infected with wild-type *L. pneumophila*, a flagellin-deficient strain of *L. pneumophila* (Δ*flaA*), Δ*flaA* expressing AnkG, or Δ*dotA* for 1 and 10 h. At each time point, DCs were fixed and stained, and consequently assays to measure uptake and formation of vacuoles containing replicating *L. pneumophila* in DCs were conducted as previously described (2).

1. Kagan JC, Roy CR (2002) Legionella phagosomes intercept vesicular traffic from endoplasmic reticulum exit sites. *Nat Cell Biol* 4:945–954.
2. Nogueira CV, et al. (2009) Rapid pathogen-induced apoptosis: A mechanism used by dendritic cells to limit intracellular replication of Legionella pneumophila. *PLoS Pathog* 5:e1000478.
3. Jantsch J, et al. (2008) Small interfering RNA (siRNA) delivery into murine bone marrow-derived dendritic cells by electroporation. *J Immunol Methods* 337:71–77.

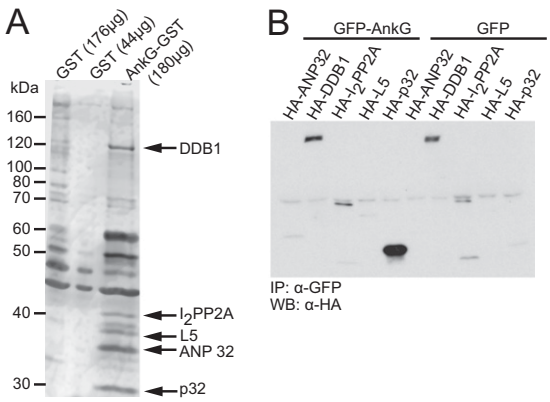

Fig. S1. Identification of host-cell proteins that bind to AnkG. (*A*) Affi-Gel columns with immobilized GST-AnkG or GST alone were incubated with lysates from CHO. Bound proteins were analyzed by SDS/PAGE and silver staining after elution. Arrows indicate the position of five unique bands that were excised and analyzed by MALDI-TOF. Protein identities as predicted by mass spectrometry are indicated next to each arrow. (*B*) HEK 293 cells were cotransfected with plasmids encoding GFP-AnkG or a control GFP along with the potential AnkG binding partners tagged with the HA epitope. Proteins in the lysates were immunoprecipitated with an anti-GFP antibody (IP) and analyzed by immunoblotting (WB) with an anti-HA antibody.

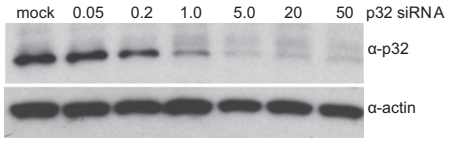

Fig. S2. siRNA silencing of p32. HeLa cells were transfected with indicated concentrations of p32 siRNA (nM). The efficiency of p32 silencing was determined 3 d posttransfection by immunoblot analysis using an anti-p32 antibody. The anti-actin immunoblot was used as a control for equal protein loading.

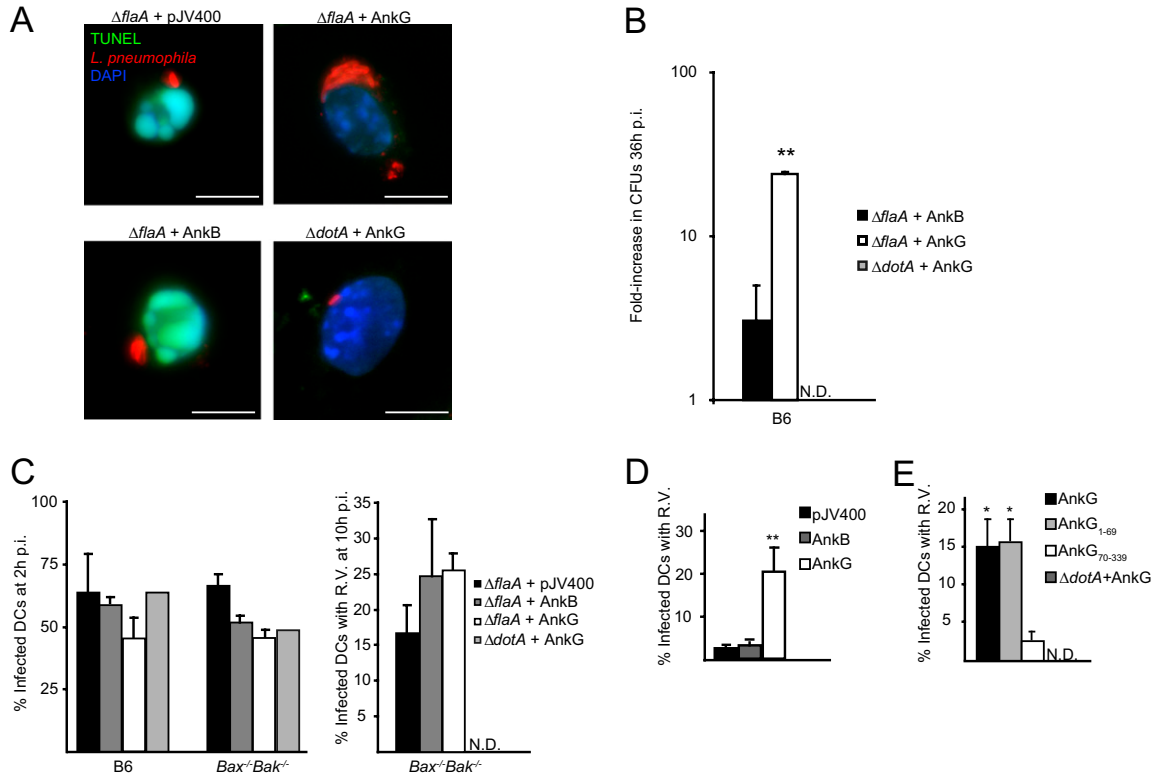

Fig. S3. The antiapoptotic effects of AnkG during *L. pneumophila* infection are sufficient to allow bacterial intracellular replication in DCs. (*A*) Fluorescence micrographs show TUNEL staining (green) of DCs derived from B6 mice infected for 6 h with *L. pneumophila* Δ*flaA* (red) expressing the pJV400 vector (Δ*flaA* + pJV400) (*Upper Left*), pJV400-AnkG (Δ*flaA* + AnkG) (*Upper Right*), pJV400-AnkB (Δ*flaA* + *AnkB)* (*Lower Left*), or *L. pneumophila* Δ*dotA* expressing AnkG (Δ*dotA* + AnkG) (*Lower Right*). Note that in the Δ*flaA* + AnkG cell there were no obvious signs of apoptosis despite the presence of a large replicative vacuole containing *L. pneumophila*. Total DNA was stained with DAPI (blue), and bacteria were stained in red. (Scale bar: 10 μm.) (*B*) B6 DCs were infected with *L. pneumophila* Δ*flaA* expressing AnkB (Δ*flaA* + AnkB) (black bars) or AnkG (Δ*flaA* + AnkG) (white bars) or with *L. pneumophila* Δ*dotA* + AnkG (gray bars) for 36 h. The efficiency of intracellular replication was determined by dividing the number of *L. pneumophila* cfus recovered at 36 h postinfection by the number of cfus recovered at 1 h postinfection. Data for each time point are the average of values obtained from three independent wells. $^{**}P < 0.01$. N.D., not detectable. (*C*) Graphical representations of the percentage of B6 and Bcl-2–associated X *(Bax)*$^{-/-}$ BCL2-antagonist/killer *(Bak)*$^{-/-}$ DCs infected at 2 h (*Left*) and the percentage of infected *Bax*$^{-/-}$*Bak*$^{-/-}$ DCs with vacuoles containing replicating bacteria at 10 h postinfection (*Right*). Data represent the mean ± SD of 300 cells counted per coverslip in triplicate. R.V., vacuoles containing replicating bacteria. There were no vacuoles containing replicating bacteria detected (N.D.) for the Δ*dotA* + AnkG strain, which would have been indicated with a light gray bar. (*D* and *E*) Graphical representations of the percentage of infected B6 DCs with (*D*) *L. pneumophila* Δ*flaA* + pJV400 (black bar), Δ*flaA* + AnkB (gray bar), and Δ*flaA* + AnkG (white bar) and (*E*) *L. pneumophila* Δ*flaA* expressing AnkG (black bar), $AnkG_{1–69}$ (light gray bar) or $AnkG_{70–339}$ (white bar). There were no vacuoles containing replicating bacteria detected (N.D.) for the Δ*dotA* + AnkG strain, which would have been indicated with a dark gray bar. Vacuoles containing replicating bacteria at 10 h postinfection were counted. Shown are mean ± SD of 300 cells counted per coverslip in triplicate in at least three independent experiments. $^{**}P < 0.05$; $^{*}P < 0.01$.

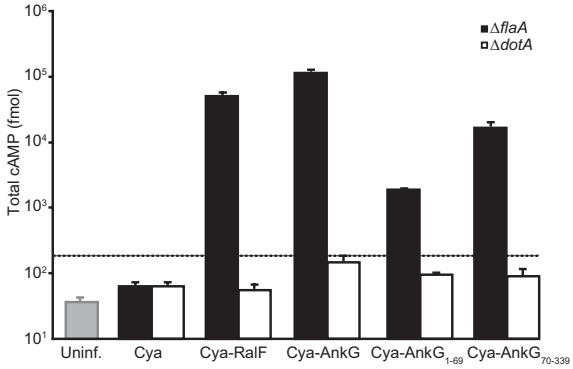

Fig. S4. $AnkG_{1–69}$ and $AnkG_{70–339}$ are translocated by the Dot/Icm secretion apparatus during *L. pneumophila* infection. CHO FcγRII cells were left uninfected (gray bars) or were infected with either *L. pneumophila* Δ*flaA* (black bars) or Δ*dotA* (white bars) expressing the indicated adenylate cyclase (Cya) fusion proteins. After 1 h of infection, tissue culture cells were lysed, and cAMP was extracted from the sample. Total cAMP (indicated as fmol) production induced by translocation of the hybrid was quantified using an enzyme-immunoassay system. Results represent average values ± SD of experiments performed in triplicate.

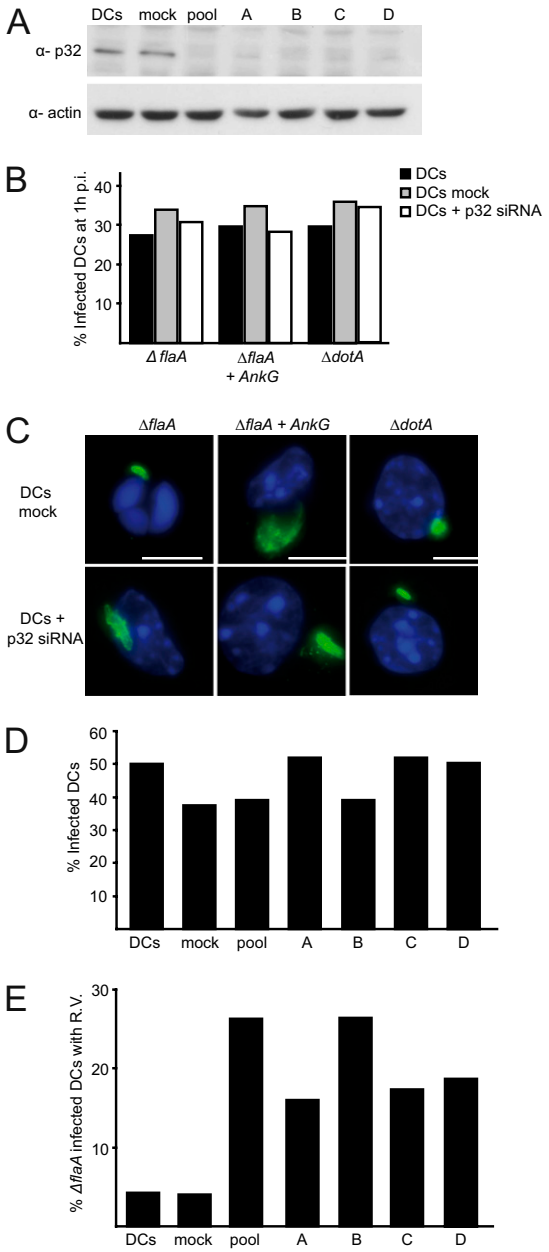

Fig. S5. Knock-down of p32 prevents *L. pneumophila*-induced apoptosis and allows bacterial intracellular replication in DCs. (*A*) DCs derived from B6 mice were electroporated with 6 μg of a pool of siRNA directed against mouse p32. The individual nonoverlapping siRNA oligonucleotides (A, B, C, D) that comprised the pool were also used to silence p32 as indicated. DCs electroporated with no siRNA were used as control (mock). Efficiency of p32 silencing was determined by immunoblot analysis 2 d after electroporation using a p32 antibody. An anti-actin immunoblot was used as a control for equal protein loading. (*B*) Shown are the percentages of untreated DCs, DCs treated with p32 siRNA, or mock-treated DCs infected with *L. pneumophila* Δ*flaA*, Δ*flaA* + AnkG, or Δ*dotA* I h postinfection. Results of one experiment representative of three independent experiments are shown. These data indicate the efficiency of bacterial uptake was not affected adversely by the siRNA treatment or by the introduction of AnkG into the bacteria. (*C*) Fluorescence micrographs show DCs either treated with siRNA against p32 or mock treated and then infected for 10 h with *L. pneumophila* Δ*flaA,* Δ*flaA* expressing AnkG, or Δ*dotA*. DNA was stained with DAPI (blue), and bacteria were stained with an anti-*Legionella* antibody (green). (Scale bar 10 μm.) (*D* and *E*) DCs were electroporated with 6 μg of siRNA single oligonucleotides (A, B, C, D) or with 6 μg of a pool of siRNA directed against mouse p32. DCs electroporated with no siRNA were used as control (mock). Two days after electroporation the DCs were sorted using CD11c magnetic beads and were infected with *L. pneumophila* Δ*flaA*. (*D*) The percentage of infected DCs at 1 h postinfection is presented for one experiment representative of three independent experiments that yielded similar results. (*E*) Vacuoles containing replicating bacteria at 10 h postinfection were counted. Data are shown for one experiment representative of three independent experiments yielding similar results. R.V., vacuoles containing replicating bacteria.

Table S1. Plasmids and primers used in this study

Plasmid	Primer*	Reference
pGEX-5X-1		Amersham
pEGFP-C1		Clontech
pJV400		This study
pJV400-*ankB*	10, 371	This study
pJV400-*ankG*	26, 373	This study
pJV400-*ankG(1-69)*	373, 389	This study
pJV400-*ankG(70-339)*	390, 26	This study
pJV400-*ankF*	22, 372	This study
pJV450-*ankG(1-69)*	25, 389	This study
pJV450-*ankG(70-339)*	402, 26	This study
pGEX-5X-2-*ankG*	96, 97	This study
pEGFP-C2-*ankA*	30, 71	This study
pEGFP-C2-*ankB*	72, 73	This study
pEGFP-C2-*ankF*	40, 74	This study
pEGFP-C-*ankG*	34, 75	This study
pEGFP-C1- p32	185, 186	This study
pCMV-HA-*ankG*	80, 307	This study
pCMV-HA-*ankG(Δ92–157)*	80, 307, 324, 325	This study
pCMV-HA-*ankG(1-69)*	329, 338	This study
pCMV-HA-*ankG(70-339)*	332, 340	This study
pCMV-HA-*anp32*	301, 302	This study
pCMV-HA-*ddb1*	312, 313	This study
pCMV-HA-i_2*pp2a*	303, 304	This study
pCMV-HA-*l5*	305, 306	This study
pCMV-HA- p32		This study
pCYA-AnkG		Pan et al., 2008 (1)
pCYA-RalF		Nagai et al., 2005 (2)
pCYA-AnkG (1-69)		This study
pCYA-AnkG (70-339)		This study

*Primer numbers are as in Table S2.

1. Pan X, Lührmann A, Satoh A, Laskowski-Arce MA, Roy CR (2008) Ankyrin repeat proteins comprise a diverse family of bacterial type IV effectors. *Science* 320:1651–1654.
2. Nagai H, et al. (2005) A C-terminal translocation signal required for Dot/Icm-dependent delivery of the *Legionella* RalF protein to host cells. *Proc Natl Acad Sci USA* 102:826–831.

Table S2. Primers used in this study

Primer	Sequence	Site
10	AA**GGCGCGCC**ttacatgtgcttacccggg	*AscI*
22	aa**ggcgcgcc**ctaccgctggaagccgc	*AscI*
25	AA**GGCGCGCC**AAGTAGACGTGAGACTCCC	*AscI*
26	AA**GGCGCGCC**tcaccgaggactagacag	*AscI*
30	AA**GGATCC**ttaaaacagtccggggcct	*BamHI*
34	AA**GGATCC**tcaccgaggactagacaga	*BamHI*
40	AA**GGATCC**ctaccgctggaagccgc	*BamHI*
71	CC**GAATTC**ttgcagttggcagcccgt	*EcoRI*
72	CCCC**TGCAG**atgtttaaccaattggaaatagt	*PstI*
73	CC**GGTACC**ttacatgtgcttacccggg	*KpnI*
74	CCCC**TGCAG**Tatgagacagcgtgaaattaat	*PstI*
75	CC**GGTACC**GCatgagtagacgtgagactc	*KpnI*
80	CC**GGTACC**tcaccgaggactagacaga	*KpnI*
96	CC**GGATCC**CCatgagtagacgtgagactc	*BamHI*
97	CCC**CCCGGG**tcaccgaggactagacaga	*SmaI*
185	CC**GGATCC**CTACTGGCTCTTGACAAAACT	*BamHI*
186	CC**GGATCC**CTGCACACCGACGGAGAC	*BamHI*
301	cc**ggtacc**atggagatgaagaagaagattaa	*KpnI*
302	cc**ggtacc**ctagtcatcttcttcctctc	*KpnI*
303	cc**ggtacc**atgtcggcgccggcgg	*KpnI*
304	cc**ggtacc**ctagtcatcttctccttcatc	*KpnI*
305	cc**ggtacc**atggggtttgttaaagttgttaa	*KpnI*
306	cc**ggtacc**ttagctctcagcagcccg	*KpnI*
307	cc**ggtacc**ATGAGTAGACGTGAGACTCC	*KpnI*
312	cc**ggtacc**atgtcgtacaactacgtggta	*KpnI*
313	cc**ggtacc**ctaatggatccgagttagct	*KpnI*
324	AGGTTCTGTCCTAAATCCGTCTTTGGCGGTAC	
325	AAAGACGGATTTAGGACAGAACCTTTTAGAACT	
329	cca**agatct**ctATGAGTAGACGTGAGACTCC	*BglII*
332	cca**agatct**ctatgCTTCGCGGGGATTCTTTTCA	*BglII*
338	cc**ggtacc**tcaGTAGTTTTTTATTATGTCTAAGCT	*KpnI*
340	cc**ggtacc**TCACCGAGGACTAGACAGA	*KpnI*
341	cc**gaattc**tatgtcgggaggtggtgtga	*EcoRI*
342	cc**tctaga**ttatgtacgagagcgagatct	*XbaI*
343	GCATTC**CTCGAG**GGCGGATTTGAATGTAGGT	*XhoI*
344	GCATTC**GCGGCCGC**GAGAGAAGCCCAGGATAGGAC	*NotI*
345	GCATTC**GCGGCCGC**CCTGACCCTGCCCATCTCGTT	*NotI*
346	GCATTC**AAGCTT**ACCAGCGGTTGAAGCGTTCCT	*HindII*
348	CCAGCGGTTGAAGCGTTCCT	
351	TGATCTAGAGTCGCGGCCGA	
353	GGAAGAGAACAGGACTGAGGC	
354	GTTTTTGTTCGGGCCCAAGCTT	
371	cc**ggccggcc**ATGTTTAACCAATTGGAAATAGT	*FseI*
372	cc**ggccggcc**atgagacagcgtgaaattaatg	*FseI*
373	cc**ggccggcc**ATGAGTAGACGTGAGACTCC	*FseI*
389	cc**ggcgcgcc**tcagtagttttttattatgtctaagc	*AscI*
390	cc**ggccggcc**atgcttcgcggggattcttttca	*FseI*
402	cc**ggcgcgcc**aatgcttcgcggggattcttttca	*AscI*

Boldface denotes the location of the restriction site indicated in the next column.