hand axes have been observed protruding from or lying directly on near-surface calichified alluvium, we believe that some late Acheulian occupations were contemporary with the last stages of aggradation along the edges of the large valleys, perhaps as late as 141 ka. At Bir Tarfawi (Fig. 1), late Acheulian remains are associated with lake beds and spring deposits inset into a broad, carbonate-cemented plain that bears many Acheulian sites (*14*). These carbonate deposits and Acheulian sites are most likely penecontemporaneous with those in Wadi Arid.

Middle Paleolithic artifacts are indirectly associated with the third carbonate deposition episode at 45 ka; they occur in freshwater deposits, and similar deposits (containing the bivalve *Corbicula*) in BHT 84-22 have been dated by ^{14}C at 40.1 ± 2.2 ka (*6*) (Fig. 2). The single U-series age of 15 ka derived from a rootcast collected from Wadi Safsaf suggests that this episode of carbonate deposition may be late Pleistocene in age (*15*). The U-series age of >300 ka derived from one calcrete sample from BHT 84-27 on the northwest edge of Wadi Arid approaches the limit of the dating technique, and it therefore is a minimum age. The U-series dates thus allow the identification of separate pluvial humid phases in the Eastern Sahara and have provided a provisional chronology of geomorphic and climatic events and cultural developments of the past 300,000 years in this region.

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11. We fragmented each carbonate sample, selected and crushed the denser, carbonate-rich pieces and cleaned them of bedrock particles (larger than fine sand size) by sieving. We ground the sample fraction that passed through a 0.1-mm sieve and heated 5- to 10-g splits for about 8 hours at 900°C to convert $CaCO_3$ to CaO. We added small portions of weighed samples to a continuously stirred dilute solution of nitric acid (0.10 to 0.25*N*) and adjusted the final acidity of the slurry of acid-insoluble, detrital material to about *p*H 2; we then separated the soluble and insoluble fractions with a centrifuge. Some of the sample aliquots (designated by "L" following the lab number in Table 1) were not ignited. We leached the calcium carbonate of these samples with dilute nitric acid (0.10 to 0.25*N*), then separated soluble and insoluble fractions with a centrifuge. We dissolved the acid-insoluble residues chosen for analyses by repeated heating with concentrated HF and $HClO_4$ mixtures (residue samples are designated by "R" at the end of the lab number in Table 1). We spiked both acid-soluble and acid-insoluble fractions with weighed amounts of ^{236}U, ^{228}Th, and ^{229}Th standard solutions. We isolated and purified U and Th isotopes by chemical procedures similar to those in B. J. Szabo, W. J. Carr, W. C. Gottschall [*U.S. Geol. Survey Open-File Rep. 81-1190* (1981)] and determined their concentrations by alpha spectrometry counting. Pure calcium carbonate typically contains a negligible amount of ^{232}Th relative to ^{238}U and ^{230}Th; therefore the measured values of $^{230}Th/^{232}Th$ ratios are larger than 10. In our carbonate samples reported, the values of $^{230}Th/^{232}Th$ ratios ranged from 1.3 to 9.7 (except sample Sch-18L as discussed in the text), indicating that ^{232}Th was removed from the residue fractions during the acid leaching procedure. Likewise, some ^{230}Th and some U were also removed during the acid treatment, modifying the true values of the activity ratios of the pure carbonates. We corrected for the effect of the acid leaching by applying a graphical correction procedure [B. J. Szabo and J. N. Rosholt in *Uranium-Series Disequilibrium: Application to Environmental Problems*, M. Ivanovich, R. W. Harmon, Eds. (Oxford Univ. Press, New York, 1982), pp. 246–267]. From these corrected activity ratio values, the U-series ages for sample groups are calculated, with the assumptions that (i) the samples were homogeneous, authigenic deposits; (ii) they remained in a closed system with respect to U and Th through time; and (iii) they incorporated U free of Th at the time of formation.
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15. Wadi Safsaf currently contains phytogenic mounds covered by *Acacia erhenbergiana* or *Tamarix nilotica*, or both, supported by groundwater that is only 2 to 3 m below the surface in some areas. Around many of these mounds are thin, modern surface crusts of $CaCO_3$ (and other salts) derived from groundwater taken up by the plant roots. Similar crusts occur in the surrounding sand sheets at depths of a few centimeters and are of Holocene or latest Pleistocene age. The last 20,000 years of the Pleistocene are generally conceded to have been hyperarid, because there is no archeological evidence of human occupation in the Eastern Sahara for that period (*14*).

16. The fieldwork was supported by the Egyptian Geological Survey and Mining Authority, the Egyptian General Petroleum Company, USAID-Cairo, and the National Aeronautics and Space Administration (NASA). We thank the Combined Prehistoric Expedition and F. Wendorf, R. Schild, and A. Close for supporting and assisting W. P. McHugh in the field in 1985. Space shuttle radar research for the U.S. Geological Survey was supported by NASA's Office of Space Applications under NASA contract WO-8760 to G.G.S. Research on the Quaternary paleoenvironmental changes was supported by NSF grant 8607479 and National Geographic Society grant 2790-84 to C.V.H. We thank P. Davis, A. L. Berlin and D. Weir for reviewing a draft of the report.

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Specific Recognition of Cruciform DNA by Nuclear Protein HMG1

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Cruciform DNA, a non–double helix form of DNA, can be generated as an intermediate in genetic recombination as well as from palindromic sequences under the effect of supercoiling. Eukaryotic cells are equipped with a DNA-binding protein that selectively recognizes cruciform DNA. Biochemical and immunological data showed that this protein is HMG1, an evolutionarily conserved, essential, and abundant component of the nucleus. The interaction with a ubiquitous protein points to a critical role for cruciform DNA conformations.

CRUCIFORM STRUCTURES ARE OF INTEREST both as sequence-dependent variations in DNA structure and as models of the transient Holliday junctions of homologous genetic recombination (*1*). Symmetric cruciform structures are inherently unstable, but certain palindromic sequences have been shown to form cruciforms under conditions of supercoiling in *Escherichia coli* (*2*). Stable nonsymmetric cruciform DNA molecules can, however, be constructed by annealing appropriately chosen sequences (*3*). We used such synthetic cruciforms to identify and purify eukaryotic proteins that could recognize and stabilize cruciform junctions. Two polypeptides from rat liver that specifically bind to cruciform DNA showed a high degree of sequence similarity to nonhistone high mobility group protein 1 (HMG1), an abundant eukaryotic nuclear protein whose function is not precisely known (*4*). Further experiments showed that HMG1 selectively recognizes cruciform DNA and suggested that the active polypeptides recovered from liver extracts are degradation products of HMG1.

We used two small synthetic cruciforms

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(molecule c, 151 nucleotides, and molecule f, 196 nucleotides) (Fig. 1) to develop an assay for cruciform-binding activities and to purify them by affinity chromatography. Rat and human cells were shown to contain a cruciform-binding protein that is structure-specific and sequence-independent (*5, 6*). The rat protein binds to the synthetic cruciform DNA on which the assay is based, but not to linear DNA molecules of identical sequence (Fig. 1). In addition, the rat protein binds to supercoiled molecules of a plasmid that contains a 70-bp palindrome, but does not bind to the linearized plasmid (*5*). No nucleolytic activity was found associated with this protein, suggesting that it serves a different function compared to the specific nucleases that cleave three- and four-branched DNAs (*7*).

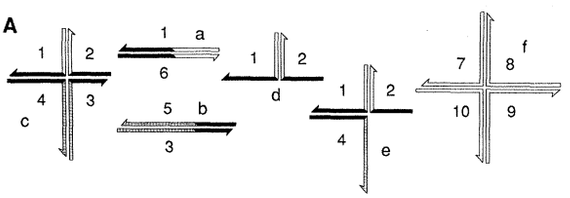
We purified the cruciform binding activity present in rat liver extracts by affinity chromatography and obtained a preparation containing only two polypeptides with apparent molecular sizes of 23.5 and 21 kD, which we called p23 and p21 (*8*). When recovered from a preparative SDS-polyacrylamide gel and renatured, both p23 and p21 showed strong cruciform-binding activity, whereas all of the other slices of the gel did not contain any activity (Fig. 2).

Tryptic digests of p23 yielded several peptides, four of which were purified and sequenced. Each of the four sequences corresponded to stretches of the protein sequence previously determined from rat HMG1 cDNA (*9*) (Fig. 3A). To confirm that the p23 cruciform-binding protein is related to HMG1, we performed a preparative bandshift experiment (Fig. 3B). The protein that forms a complex with cruciform DNA was recognized by rabbit antibodies to HMG1; preimmune antibodies from the same rabbit did not react. Since p21 reacted in a like manner and was recovered in small and variable amounts, we assumed that the protein was a degradation product of p23 and did not analyze it further.

HMG1 and p23 are not identical, however, since the former has a predicted molecular size of 24.9 kD and migrates in SDS-polyacrylamide gels with an apparent molecular size of 27 kD (*4, 9*). Immunoblots of proteins extracted from various rat organs by a rapid procedure did not provide evidence of the presence of immunoreactive species smaller than 27 kD. The analysis of samples taken at different stages during the purification of the cruciform-binding proteins revealed that the immunoreactive polypeptide of 27 kD was completely converted to smaller sizes within a few hours after the salt-extraction of nuclei. Thus, p23 probably is not present in vivo, but forms during the purification procedure by proteolytic cleavage of HMG1.

To test whether intact HMG1 can bind to cruciform DNA, we prepared HMG1 from rat liver according to a protocol that exploits its high solubility in concentrated ammonium sulfate (*10*), and the preparation displayed cruciform-binding activity. As a final test, we synthesized ^{35}S-labeled HMG1 in vitro from the cloned rat cDNA (Fig. 4A). More than 95% of the translation products comigrated with authentic HMG1 protein and a small fraction comigrated with p23, suggesting that proteolysis of HMG1 occurred also in in vitro translation extracts. Preliminary tests showed that the products synthesized from the HMG1 transcript bound to cruciform DNA, whereas the products of the control translations did not. We purified the larger polypeptide synthesized from the HMG1 transcript away from most of the rabbit reticulocyte proteins and the smaller labeled impurities, and assayed it for binding to cruciform DNA (Fig. 4B). Most of the labeled HMG1 protein, when loaded in the absence of any DNA, did not migrate far into the gel (Fig. 4B, lane 1). In the presence of sonicated salmon sperm DNA, most of the protein remained in the well, but a distinct band formed between the well and the position where the bulk of the DNA had migrated (lane 2). Previous results suggested that sonicated salmon sperm DNA contains DNA structures weakly recognized by the cruciform-binding protein (*5*). When incubated in the presence of competitor salmon sperm DNA and cruciform DNA, a sizable fraction of the labeled protein migrated to a defined position behind the band of naked cruciform DNA (lane 5). However, HMG1 formed no complexes with control duplexes (lanes 3 and 4).

These results indicate that full-length HMG1 can bind to cruciform DNA with high affinity and suggest that p23 corre-



B

1. CCCTATAACCCCTGCATTGAATTCCAGTCTGATAA
2. GTAGTCGTGATAGGTGCAGGGGTTATAGGG
3. AACAGTAGCTCTTATTCGAGCTCGCGCCCTATCACGACTA
4. TTTATCAGACTGGAATTCAAGCGCGAGCTCGAATAAGAGCTACTGT
5. GTAGTCGTGATAGGGCGCGAGCTCGAATAAGAGCTACTGT
6. TTTATCAGACTGGAATTCAATGCAGGGGTTATAGGG
7. AATCTTTGATTTCCAAGTCGGACAATGCATCGGCAGCAGCTTTGGA
8. AAGGAGGAGGCCAAGGAAGAGGAGGTTGTCCGACTTGGAAATCAAAGATT
9. CCGAATCCCATATCTTCATCAGACTCCTCCTCTTCCTTGGCCTCCTCCTT
10. TTCCAAAGCTGCTGCCGATGCAAGTCTGATGAAGATATGGGATTCGG

Fig. 1. Design of artificial cruciform and control DNAs. (**A**) Molecules c and f, unlike natural cruciform DNAs, have no sequence symmetry and cannot dissociate through branch migration. These two artificial cruciforms have no obvious sequence similarity, but are both recognized by the cruciform-binding protein; molecules a and b contain exactly the same sequence as the four arms of molecule c, but are not recognized. Binding is thus sequence-independent but structure-specific. In addition, the rat cruciform-binding protein does not bind to hairpin-like structures such as molecule d or to single strands with no secondary folding, but binds weakly to structures of the type indicated as molecule e (*5, 16*). Cruciform and linear duplex DNA molecules were produced by annealing chemically synthesized oligonucleotides (*3, 5*). Molecule c was labeled by treating one of the constituent oligonucleotides with T4 polynucleotide kinase and was used as the probe in the assay for protein binding. Molecule f was coupled to Sepharose beads and was used as an affinity ligand for the purification of cruciform-binding proteins. (**B**) The sequences of the oligonucleotides represented in the drawing are shown.

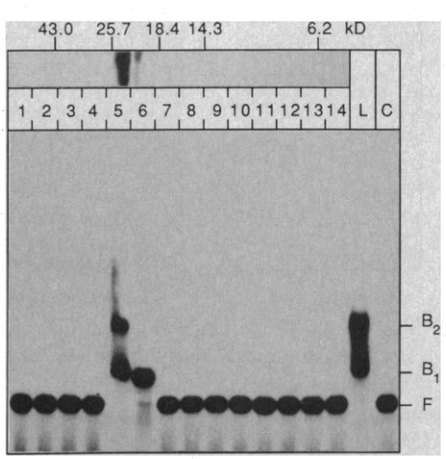


Fig. 2. Purification and identification of the polypeptides endowed with cruciform DNA-binding activity. Cruciform-binding proteins were purified from rat liver as described (*8*). A portion of the most purified fraction was separated by electrophoresis on a 15% SDS-polyacrylamide gel. One part of the gel was stained with Coomassie blue (upper horizontal lane), and the other part was cut in 14 slices, as indicated under the stained lane. The proteins contained in the individual slices were recovered, renatured, and then assayed for cruciform DNA-binding activity (*17*). Lanes 1 to 14, lower part of the figure: samples recovered from gel slices 1 to 14; lane L, sample from the first round of affinity chromatography; and lane C, control with no protein added. F indicates the position of free cruciform DNA; B_1, the position of the DNA complexed to cruciform-binding proteins p23 or p21; and B_2, the position of the complex presumably formed by one molecule of cruciform DNA with several molecules of cruciform-binding protein (*16*).

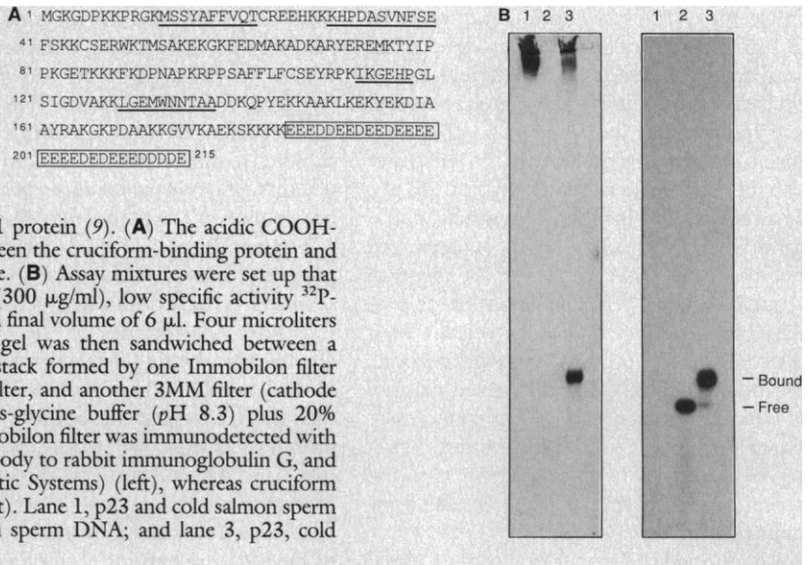


Fig. 3. The protein that binds cruciform DNA is related to HMG1. Protein p23 was subjected to partial tryptic hydrolysis and the deriving oligopeptides were purified by high-performance liquid chromatography (HPLC). Four peptides were partially sequenced with a gas-phase sequencer (*18*), yielding the sequences XGEXXNNTAA, IKGEXP, KHPDASVNFSE, and MSSYAFFVQT (underlined). X represents undetermined amino acids. A search in the European Molecular Biology Laboratory (EMBL) Data Library indicated that all four peptides were contained in the deduced sequence of rat HMG1 protein (*9*). (**A**) The acidic COOH-terminus of the protein is boxed. To confirm the similarity between the cruciform-binding protein and HMG1, we showed that they are immunologically cross-reactive. (**B**) Assay mixtures were set up that contained, in various combinations, cold salmon sperm DNA (300 μg/ml), low specific activity ^{32}P-labeled cruciform DNA (50 μg/ml), and p23 (~100 μg/ml) in a final volume of 6 μl. Four microliters of these mixtures were analyzed by gel electrophoresis. The gel was then sandwiched between a Whatman 3MM filter soaked in 1% SDS (anode side) and a stack formed by one Immobilon filter (Millipore), one Whatman 3MM filter, one Whatman DE-81 filter, and another 3MM filter (cathode side). Electroblotting was carried out overnight in 0.2*M* tris-glycine buffer (*p*H 8.3) plus 20% methanol. After electroblotting, the protein retained on the Immobilon filter was immunodetected with RPA-199 antibody (*19*), biotin-conjugated secondary goat antibody to rabbit immunoglobulin G, and avidin-conjugated alkaline phosphatase (Research and Diagnostic Systems) (left), whereas cruciform DNA was localized by autoradiography of the DE-81 filter (right). Lane 1, p23 and cold salmon sperm DNA; lane 2, labeled cruciform DNA probe and cold salmon sperm DNA; and lane 3, p23, cold salmon sperm DNA, and labeled cruciform DNA probe.

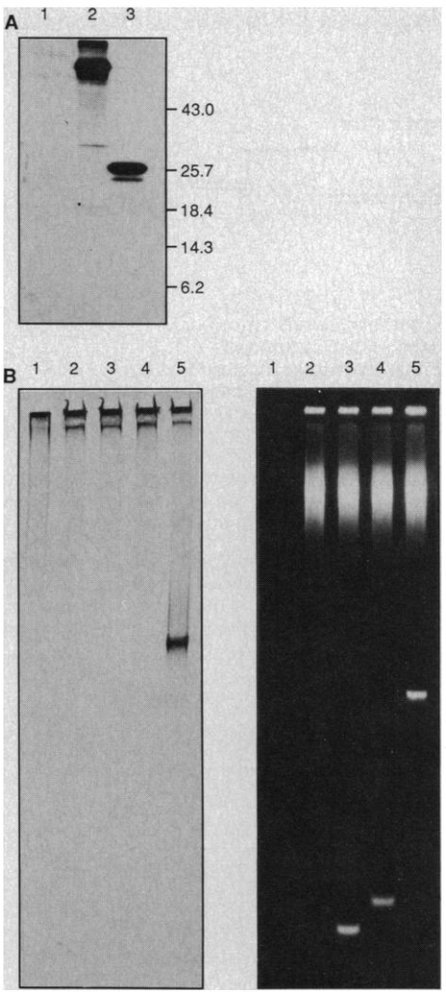


Fig. 4. In vitro synthesized HMG1 protein binds selectively to cruciform DNA. Plasmid pRNHMG1 was constructed by subcloning nucleotides −6 to 787 from the cDNA coding for rat HMG1 (*9*) between the Eco RI and Sma I sites of plasmid pTZ18R (Pharmacia). The Bam HI–cut plasmid was transcribed with T7 RNA polymerase as recommended by the manufacturer (Stratagene). The transcript was translated in vitro by a rabbit reticulocyte lysate (Promega) with ^{35}S-labeled Met as described by the manufacturer. Control translations were programmed with an equivalent quantity of brome mosaic virus RNA (Promega) or no added RNA transcript. A portion of the translation products was analyzed by electrophoresis on a 12.5% SDS-polycrylamide gel. (**A**) Lane 1, no added transcript; lane 2, translation products of the HMG1 transcript. Labeled HMG1 was then purified by gel chromatography on Sephacryl S-100 Superfine (Pharmacia) equilibrated in buffer D (*8*) containing 100 m*M* KCl, concentrated with a Centricon cartridge (Amicon), and assayed for DNA binding activity. (**B**) Approximately 1.5 × 10^4 cpm of ^{35}S-labeled HMG1 were added to assay mixtures containing, in a final volume of 8 μl, no DNA (lane 1), sonicated salmon sperm DNA (150 μg/ml) (lane 2), salmon sperm DNA (150 μg/ml) plus control duplex A (30 μg/ml) (lane 3), control duplex B (lane 4), or cruciform DNA C (lane 5). After electrophoresis, the gel was briefly stained with ethidium bromide to visualize the DNA (right) and was then electroblotted onto an Immobilon filter, which was dried and autoradiographed (left) to visualize the labeled protein.

sponds to a fragment of HMG1 arising by proteolysis during purification. HMG1 binds to duplex DNA with low affinity and with somewhat higher affinity to single-stranded DNA (*11*) and to a plasmid DNA that could potentially form supercoil-induced secondary structures (*12*). The results obtained with incomplete cruciform structures (Fig. 1, molecule e) (*5*) suggest that the apparent specificity toward single-stranded DNA is due to the presence of secondary structures, reminiscent of branched duplex DNA, in long DNA single strands.

The physiological significance of the specificity of binding of HMG1 toward branched DNA is at present unknown. HMG1-like proteins are present in all eukaryotes and are apparently essential for cell viability (*13*), but their function has not been identified unequivocally. Mammalian HMG1 has been implicated in transcription and in DNA replication (*4*): both of these processes can generate branched DNA molecules either directly or through the action of induced supercoiling. Our results imply that whenever DNA adopts a cruciform conformation, the high abundance of HMG1 in cell nuclei (*4*) can effectively drive the equilibrium toward the formation of a protein-DNA complex. Whether the formation of such complexes is passive (protection of the "alternative" structures until disposal by additional nuclear activities) or serves a specialized function is not known. However, HMG1 has a remarkable domain structure, with a DNA-binding positively charged core and a long COOH-terminal string of acidic residues (*14*). Protein surfaces of localized high negative charge have been implicated in protein-protein interactions (*15*) and the acidic domain of HMG1 protein may have a similar function.

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19. Antibody RPA-199 was obtained as follows. HMG1 (300 μg) purified from rat liver (*10*) was separated by electrophoresis on a 15% SDS-polyacrylamide gel. The gel slice containing HMG1 was excised after staining with Coomassie blue, minced, and administered subcutaneously with Freund's adjuvant to a New Zealand White (NZW) rabbit over 3 weeks. The animals serum was incubated with 300 μg of HMG1 blotted onto a strip of Immobilon; the strip was washed extensively with TBSN (50 m*M* tris-HCl, *p*H 8.0, 150 m*M* NaCl, and 0.1% nonidet P-40); the specific antibodies were eluted with 0.1*M* glycine-HCl, *p*H 2.5, and immediately neutralized. Affinity-purified RPA-199 reacted with a single band with an apparent molecular size of 27 kD on blots of total extracts of rat, mouse, and human cells.
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A *Salmonella* Locus That Controls Resistance to Microbicidal Proteins from Phagocytic Cells

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Facultative intracellular pathogens pose an important health problem because they circumvent a primary defense mechanism of the host: killing and degradation by professional phagocytic cells. A gene of the intracellular pathogen *Salmonella typhimurium* that is required for virulence and intracellular survival was identified and shown to have a role in resistance to defensins and possibly to other microbicidal mechanisms of the phagocyte. This gene may prove to be a regulatory element in the expression of virulence functions.

PROFESSIONAL PHAGOCYTIC CELLS are important in the body's defense against pathogenic microorganisms; they engulf and kill invading microbes by both oxygen-dependent and oxygen-independent mechanisms. The phagocyte undergoes a burst of respiration upon initial contact with the invading organism and produces toxic metabolites of oxygen such as hydrogen peroxide and superoxide (*1*). Oxygen-independent mechanisms include acidification of the phagosome (the membrane-lined vacuole in which the ingested microorganisms are enclosed) and degradation of its contents by proteins or peptides with antimicrobial activity; these antimicrobial substances are released from cytoplasmic granules that fuse with the phagosome to form a phagolysosome (*2, 3*). Facultative intracellular pathogens avoid killing by phagocytic cells and can persist in the host, with debilitating diseases often the result (*4*). Survival mechanisms used by intracellular pathogens include inhibition of the respiratory burst, inhibition of phagolysosome fusion, and survival within or escape from the phagolysosome (*3*). The molecular basis of these survival strategies remains largely unknown. *Salmonella typhimurium* is a facultative intracellular pathogen of mice that has been extensively used as a model system for human typhoid, a worldwide health problem, with 12.5 million cases annually (*5*). In this report, we describe a *Salmonella* gene that is necessary for virulence in the mouse and survival in the macrophage in vitro, and we show that the gene is required for resistance to defensins, microbicidal peptides found in neutrophils and macrophages. These results may have clinical relevance since patients with "specific granule deficiency," who have frequent and severe infections, almost completely lack defensins (*6*).

A molecular genetic analysis of intracellular survival was started in our laboratory with the isolation of 83 Tn*10* insertion mutants of *S. typhimurium* that were unable to survive in mouse peritoneal macrophages in vitro. All of the mutant strains were subsequently shown to be attenuated in the mouse (*7*). A subset of these mutants, represented by MS4252s, MS5996s, and MS7953s (*8*), were of particular interest in that (i) they had the highest median lethal dose (LD_{50}) of all the mutants isolated (10^5 organisms compared to <10 for the parent strain by intraperitoneal injection of BALB/c mice) (*9*); (ii) they did not persist in the mouse (no bacteria were detectable 72 hours after injections), they did not stimulate a significant humoral or cell-mediated immune response, and they did not protect a mouse against challenge with the virulent parent strain. This is in contrast to other avirulent Tn*10* mutants isolated in the same study that persisted for up to 2 months and stimulated T cell and B cell responses as well as protective immunity against challenge with virulent strains (*10*). These results suggested that the mutants may be defective in an important virulence determinant.

We investigated the possibility that these mutants were more sensitive to specific antimicrobial mechanisms of the phagocyte. Crude extracts from human neutrophils and rabbit peritoneal macrophages had a strong microbicidal effect on these mutants (Fig. 1, A and B). The sensitivity to granule extracts was not due to a lipopolysaccharide (LPS) defect, since all the mutants showed the same phage sensitivity profile and had a complete LPS (smooth) identical to that of the parent strain when purified and visualized on polyacrylamide gel electrophoresis (PAGE) (*9*). In an attempt to identify a specific component of the extract to which the mutants were sensitive, we fractionated the rabbit extract on an anion exchange column and tested the mutants with individual fractions. Those fractions that were most active against the mutants contained low

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