tional ANCOVAs overestimated the level of population differentiation because a significant allometric effect of size on burst speed was detected inconsistently in these analyses in contrast to analyses based on the full range of size-manipulated hatchlings [$F(1,56) = 6.48$, $P = 0.01$, $n = 58$] (Fig. 2C).
18. Hindlimb span was shorter for northern hatchlings [ANCOVA: factor for population $F(2,199) = 118.6$, $P << 0.01$; covariate for size, $F(2,199) = 205.0$, $P << 0.01$, difference in slopes, ns). However, some of the difference between the hindlimb span of northern and southern populations arises from an effect of yolk mass on morphology [ANCOVA comparing unmanipulated California hatchlings with experimentally miniaturized hatchlings and their full-sized sibs indicates that yolk volume has a significant effect on morphology, slopes were significantly different between these groups, $F(1,73) = 5.95$, $P > 0.02$] (Fig. 2A).
19. B. Sinervo and L. R. McEdward [*Evolution* **42**, 885 (1988)] list some vertebrates and invertebrates in which larval or juvenile size can be manipulated.
20. R. D. Palmiter, G. Norstedt, R. E. Gelinas, R. E. Hammer, R. L. Brinster, *Science* **222**, 809 (1983).
21. Differences in size are often implicated as mechanistic correlates of interpopulational or interspecific differences [S. J. Gould, *Evolution* **28**, 191 (1974)], but these implications are statistical inferences only.
22. G. F. Oster, N. Shubin, J. D. Murray, P. Allberch, *Evolution* **42**, 862 (1988).
23. J. A. Endler, *Natural Selection in the Wild* (Princeton Univ. Press, Princeton, NJ, 1986); T. Mitchell-Olds and R. G. Shaw, *Evolution* **41**, 1149 (1987); D. Schluter, *ibid.* **42**, 849 (1988).
24. The work was supported in part by funding from NSF (BSR8718063 to R.B.H.) and the Miller Institute for Basic Research in Science, University of California, Berkeley (B.S.). We thank J. B. Losos for comments on earlier versions of the manuscript.

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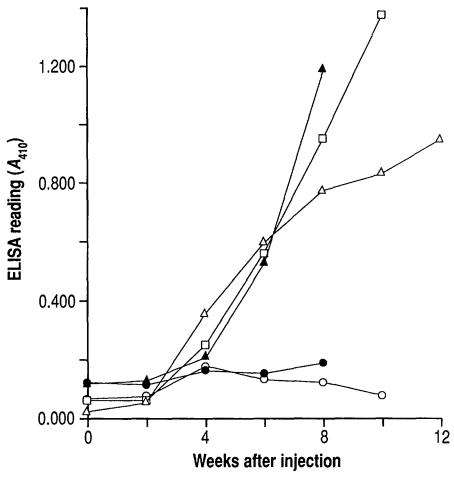

Fig. 1. Antibody responses in rhesus monkeys inoculated with SIVmac239 cloned virus. Portions of plasma from blood samples were frozen at −70°C on the weeks after inoculation and analyzed at a 1:20 dilution for antibodies to SIV by enzyme-linked immunosorbent assay (ELISA) as previously described (*6, 9, 19*). The five animals shown were inoculated with virus produced in macaque PBLs (*8*). The symbols used to identify the rhesus monkeys are ○, 316-85; ●, 452-87; □, 54-83; △, 326-87; and ▲, 124-79.

Induction of AIDS in Rhesus Monkeys by Molecularly Cloned Simian Immunodeficiency Virus

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Better understanding of the pathogenesis of acquired immunodeficiency syndrome (AIDS) would be greatly facilitated by a relevant animal model that uses molecularly cloned virus of defined sequence to induce the disease. Such a system would also be of great value for AIDS vaccine research. An infectious molecular clone of simian immunodeficiency virus (SIV) was identified that induces AIDS in common rhesus monkeys in a time frame suitable for laboratory investigation. These results provide another strong link in the chain of evidence for the viral etiology of AIDS. More importantly, they define a system for molecular dissection of the determinants of AIDS pathogenesis.

IDENTIFICATION OF THE GENETIC determinants of oncogenicity and tissue specificity of type C retroviruses has been achieved largely through the use of cloned DNA capable of yielding pathogenic virus (*1*). Human immunodeficiency virus (HIV), the causative agent of AIDS, is a member of the lentivirus subfamily of retroviruses. Although much has been learned about the molecular biology of HIV, systems for study of disease induction by molecularly cloned HIV have not been developed. In fact, there have been no previous reports of disease induction by a molecularly cloned lentivirus from any species.

The simian immunodeficiency viruses (SIVs) are nonhuman primate lentiviruses that are the closest known relatives of HIV-1 and HIV-2. They closely parallel their human counterparts in genetic organization and biological properties (*2*). Similarities between HIV and SIV include lentiviral morphology; tropism for CD4 lymphocytes and macrophages; extra genes called *tat, rev, vip, vpr*, and *nef* that other retroviruses do not have; use of the CD4 molecule for receptor; cytopathicity; and the ability to cause chronic disease after long-term persistent infection. Infection of common rhesus monkeys (*Macaca mulatta*) with some isolates of SIV results in AIDS and death in a time frame suitable for laboratory investigation (*3*). Features of the AIDS-like disease induced by SIV include CD4 lymphocyte depletion, opportunistic infections, severe weight loss, opportunistic neoplasms, and a multifocal granulomatous encephalitis. These are also features characteristic of HIV-induced disease in humans. The similarity in genomic organization, the extensive sequence homology, and the similarity in biological properties both in vitro and in vivo suggest that SIV systems are highly suitable for study of the mechanisms and determinants of HIV-induced disease.

In previous studies, three infectious molecular clones of SIV from macaque monkeys (SIVmac) were characterized (*4–6*). Of these three clones, SIVmac239 appeared to be most natural in that it grew best in primary cultures of macaque peripheral blood lymphocytes (PBLs) (*6*) and it retained a full-length 41-kD transmembrane protein rather than the truncated forms that result from growing SIV in human cells (*7*). We thus pursued in greater detail the pathogenic potential of this molecular clone.

SIVmac239-cloned DNA was transfected into primary macaque PBLs and into Hut 78 cells (a human $CD4^+$ T cell line) by a DEAE-dextran procedure, and stock virus was frozen for subsequent animal inoculations (*8*). To be useful for future mutagenesis experiments, it was important that we tested not only the original SIVmac239 lambda DNA clone but also plasmid subclones. We, and others, have experienced considerable difficulty in subcloning the full-length proviral DNA into plasmid vectors. We thus subcloned two segments of the DNA separately and then ligated them at their common restriction site before transfection (*8*).

Rhesus monkeys were inoculated with this cloned virus both at the New England Regional Primate Research Center (NERPRC) and at the California Regional Primate Research Center (CRPRC). In

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both studies, approximately 50% of the rhesus monkeys died with characteristic SIV-induced immunodeficiency disease within 1 year of infection. Fatal disease was induced in both juvenile and adult rhesus monkeys.

Stock virus produced by transfection of macaque PBLs with lambda or plasmid DNA clones of SIVmac239 was inoculated intravenously into a total of five rhesus monkeys at NERPRC. Blood samples were taken periodically from these inoculated animals and used for analysis. SIV was recovered from peripheral blood mononuclear cells (PBMC) of all five by cocultivation with CEMx174 cells on at least five separate occasions; thus, all five macaques became infected. Antibody responses were also monitored since the strength of the antibody response can be predictive of the outcome of infection. Animals with little or no antibody response are usually the first to die with AIDS after infection (*9*). Of the five inoculated rhesus monkeys, two had little or no antibody response (Fig. 1) and were thus predicted to die with AIDS 3 to 6 months after inoculation. These two animals were in fact the first to die, one at 3.5 months and the other at 5 months after inoculation. One of these rhesus monkeys (316-85) received virus derived from the lambda clone and the other (452-87) received virus derived from the plasmid subclones. Both these animals fit the pattern that we have seen previously for acute SIV-induced AIDS (*10*), since they died with features similar to HIV-induced disease in humans without ever mounting a strong antibody response to the infecting SIV.

Rhesus monkey 316-85 became critically ill and was killed 168 days after being inoculated with molecularly cloned SIVmac239. Beginning 2 months after inoculation this animal developed a cutaneous rash that recurred intermittently until the time of death. During the course of its illness the animal developed severe diarrhea, lost 15% of its body weight, and terminally had clinical evidence of bacteremia. On postmortem examination, it had a severe, purulent, and necrotizing bacterial enterocolitis; generalized Gram-negative bacteremia; marked lymphocytic depletion of peripheral and visceral lymph nodes; characteristic SIV-induced giant cell pneumonia; and SIV-induced granulomatous encephalomyelitis with multinucleate giant cells (Fig. 2A).

Rhesus monkey 452-87 became clinically moribund and was euthanized 112 days after being inoculated with molecularly cloned SIVmac239. This animal lost 17% of its body weight during the course of its illness. Postmortem examination revealed thymic atrophy, depletion of lymphocytes in peripheral and visceral lymph nodes, chronic gastroenteritis with atrophy and blunting of intestinal villi, mild biliary fibrosis, and disseminated cytomegalovirus (CMV) infection involving the retroperitoneal lymph nodes, colon, and heart (Fig. 2B). The pathologic findings in these two animals are similar to those that we have previously reported in animals infected with uncloned SIVmac (*10*).

Six rhesus monkeys were also inoculated at the CRPRC with molecularly cloned SIVmac239 produced in human $CD4^+$ tumor cell lines (*8*). All six became infected since SIV was repeatedly isolated from their peripheral blood. Of the six monkeys inoculated, three died with AIDS by 11 months after inoculation (*8*). Rhesus monkey 19788 lost 20% of its body weight during the course of its illness and was euthanized 142 days after inoculation. Postmortem examination revealed widely disseminated CMV infection with involvement of the central and peripheral nervous system, thymic atrophy, lymphoid hyperplasia, necrotizing gingivitis, and pyogranulomatous enteritis. Two additional rhesus monkeys experimentally infected with molecularly cloned SIVmac239 died at 310 days after inoculation. These two lost 13 and 16% of their body weights before death. The pathologic findings in these two animals were very similar and included *Pneumocystis carinii* pneumonia; cryptosporidiosis of the intestine, biliary, and pancreatic ducts; generalized lymphoid depletion; and disseminated CMV infection (Fig. 2, C and D). In addition, rhesus monkey 17504 also had intestinal trichomoniasis and suppurative hepatitis. These results show that cloned virus produced in continuous human T cell lines, at least over short-term culture, is also pathogenic in rhesus monkeys. SIV is responsible for the induced disease since it systematically follows infection with the virus (*3, 9, 10*) and high levels of SIV expression are detected in affected tissues (*11–13*).

Cloned SIVmac239 does not replicate appreciably in rhesus monkey primary alveolar macrophage cultures. Procedures for preparation and maintenance of these macrophage cultures have been described (*11*). A lung lavage taken just before the death of 316-85 revealed numerous SIV-infected macrophages and giant cells by immunohistochemical staining (Fig. 3). Virus recovered from the lung macrophages of 316-85, in contrast to the parent virus, replicated very well in primary alveolar macrophage cultures. Although the recovered virus replicated to yield over 100,000 cpm per milliliter of reverse transcriptase activity in cell-free supernatant 7 to 14 days after infection of these macrophages, the cloned SIVmac239 virus yielded less than 10,000 cpm per milliliter of activity even after 30 days of

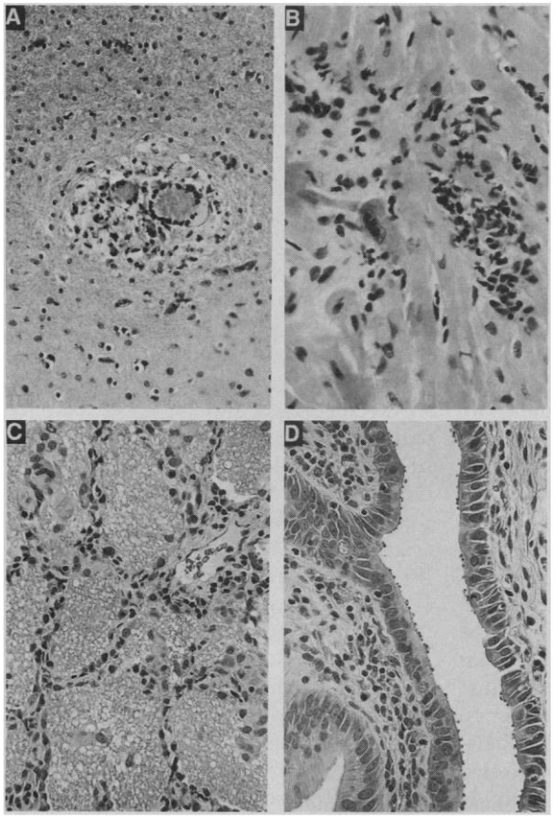

Fig. 2. Pathology findings. Tissue sections were stained with hematoxylin and eosin. (**A**) Focal granulomatous encephalitis (316-85). Aggregates of histiocytes and two multinucleate giant cells make up this SIV-induced lesion. (**B**) Focal purulent and necrotizing myocarditis with a large CMV intranuclear inclusion body in an adjacent myocyte (452-87). (**C**) *Pneumocystis carinii* pneumonia (18955). Alveoli contain abundant, foamy, pale eosinophilic material within which can be seen the outlines of small spherical structures with pale, basophilic dots characteristic of *Pneumocystis carinii*. Gomori's methenamine-silver stain and immunohistochemistry with an antibody to *Pneumocystis carinii* confirmed the diagnosis. (**D**) Cryptosporidiosis of gall bladder (18955).

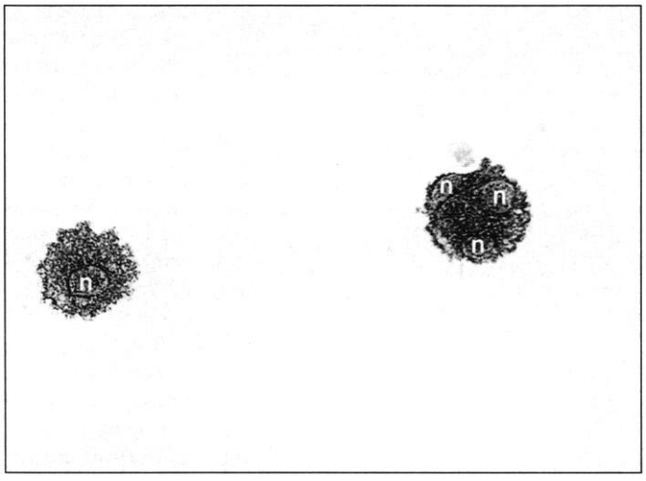

Fig. 3. Alveolar macrophages obtained by bronchoalveolar lavage from rhesus monkey 316-85 are immunoreactive for SIV core protein (p27). A three-layer peroxidase-antiperoxidase technique, performed as described previously for SIV *gag* protein (*11, 12*), was used on cytocentrifuged pulmonary cells. Intense cytoplasmic reaction product is localized to macrophages containing single and multiple nuclei (n). The cells were counterstained with the nuclear stain Mayer's hematoxylin.

culture. The recovered virus (SIVmac239/316) replicated in both lymphocytes and macrophages, whereas the parent virus (SIVmac239) replicated primarily in lymphocytes. Animal 316-85 was the only one of the five who have died so far that exhibited giant cell pneumonia, rash, and the granulomatous encephalitis. Previous studies with rhesus monkeys infected with uncloned SIV and with people infected with HIV have shown that the infected cells in these lesions are macrophages or macrophage-related cells (*14*). In 316-85, giant syncytial cells in the lung were confirmed to be SIV-infected since they stained positively with a monoclonal antibody to p27 (Fig. 3), and they were of macrophage origin since they stained positively with EBM11, a monoclonal antibody to CD68. Continued study with these materials should provide insights into the molecular determinants of macrophage tropism and its significance for the manifestations of disease.

In previous studies with uncloned stocks of SIVmac, infected rhesus monkeys with strong immune responses remained persistently infected and greater than 90% of them eventually succumbed with AIDS 1 to 3 years after infection (*9, 15*). Rhesus monkeys infected with cloned SIVmac239 who had strong antibody responses also appear to be persistently infected, since we have recovered SIV from PBMC as long as 10 months after inoculation. It seems likely that the vast majority of remaining rhesus monkeys that received the SIVmac239 cloned virus and are still alive will also develop this protracted form of disease. In fact, this longer, chronic course probably more closely approximates the usual course of HIV-induced disease in humans. However, for many laboratory applications it would be highly desirable to have a reproducible system in which close to 100% of inoculated animals die within 6 months of infection. It should be possible to achieve this goal by selecting susceptible animals or by other subtle manipulations in the experimental design. It has been suggested that rhesus monkeys whose PBLs are most permissive for SIV replication in vitro tend to be the animals who succumb most rapidly with SIV-induced AIDS (*16*). Also, as we learn more about the contributions of individual gene products, epitopes, and regulatory regions to pathogenic potential, it may be possible to identify more pathogenic forms of this cloned virus.

Viral DNA within the pathogenic plasmid subclones has been completely sequenced. The SIVmac239 sequence as well as flanking cellular sequences have been filed with GenBank database under accession number M33262. The proviral genome, including both long terminal repeats (LTRs), is 10,279 bp in length. Open reading frames for *gag, pol, env, tat, rev, vip, vpr, vpx*, and *nef* are present. The sequence is highly related to those previously published for other SIVmac clones (*4, 17, 18*); for example, the percent amino acid identities in *gag, pol, env* (outer membrane protein), and *env* (transmembrane protein) when compared to SIVmac142 (*4*) are 95.3%, 97.3%, 90.4%, and 93.6%, respectively. Among the several sequence differences in the LTR are a single base transition in one of the Sp1 binding sites and a single base pair insertion downstream of the TATA box. The reading frame for the transmembrane protein is fully open and produces a 41-kD polypeptide in macaque PBL cultures (*7*). A premature stop codon in *nef* at amino acid 93 truncates an otherwise open *nef* gene. In spite of the high degree of sequence similarity to the SIVmac239 clone, SIVmac142 cloned virus does not replicate appreciably in macaque PBLs in vitro and does not infect rhesus monkeys in vivo (*6*). Also, SIVmac251 and 1A11 cloned viruses have not induced disease in eight rhesus monkeys after more than 1 year of observation. Further work will be needed to identify the molecular determinants of these differences.

Our results show that molecularly cloned SIV can induce in common rhesus monkeys a disease remarkably similar to AIDS in humans in a time frame suitable for laboratory investigation. The SIVmac239 clone will be useful for basic research into the molecular mechanisms underlying disease pathogenesis, for drug development, and for AIDS vaccine research. Issues of immediate importance include the roles of the so-called "nonessential" genes, individual protein epitopes, and regulatory sequences in viral persistence, cell specificity, disease characteristics, and pathogenic potential. Better understanding of the basic mechanisms underlying pathogenesis will eventually fuel the drug and vaccine development effort.

REFERENCES AND NOTES

1. Y. Li, E. Golemis, J. W. Hartley, N. Hopkins, *J. Virol.* **61**, 693 (1987); E. Golemis, Y. Li, J. W. Hartley, N. Hopkins, *ibid.* **63**, 328 (1989); Y. Paquette *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 3896 (1989); H. L. Robinson, L. Jensen, J. M. Coffin, *J. Virol.* **55**, 752 (1985).
2. R. C. Desrosiers, *Annu. Rev. Microbiol.* **42**, 607 (1988); ——— and D. J. Ringler, *Intervirology* **30**, 301 (1989); R. C. Desrosiers, *Annu. Rev. Immunol.* **8**, 557 (1990); ———, M. D. Daniel, Y. Li, *AIDS Res. Hum. Retroviruses* **5**, 465 (1989).
3. M. D. Daniel *et al.*, *Science* **228**, 1201 (1985); N. L. Letvin *et al.*, *ibid.* **230**, 71 (1985); G. B. Baskin, M. Murphey-Corb, E. A. Watson, L. N. Martin, *Vet. Pathol.* **25**, 456 (1988); R. E. Benveniste *et al.*, *J. Virol.* **62**, 2091 (1988); P. N. Fultz, H. M. McClure, D. C. Anderson, W. M. Switzer, *AIDS Res. Hum. Retroviruses* **5**, 397 (1989).
4. L. Chakrabarti *et al.*, *Nature* **328**, 543 (1987).
5. H. W. Kestler III *et al.*, *ibid.* **331**, 619 (1988).
6. Y. M. Naidu *et al.*, *J. Virol.* **62**, 4691 (1988).
7. T. Kodama *et al.*, *ibid.* **63**, 4709 (1989).
8. The isolation of the recombinant SIVmac239 lambda clone has been described previously (*6*). A plasmid subclone from an Sph I site in left flanking cellular sequences to the Sph I site at viral nucleotide number 6451 was generated in cloning vector pBS(+) to yield p239SpSp5′. A plasmid subclone from the Sph I site at nucleotide 6452 to an Eco RI site in right flanking cellular sequences was generated in cloning vector pBS(−) to yield p239SpE3′. The inserts are 6706 bp in p239SpSp5′ and 6361 bp in p239SpE3′. p239SpSp5′ and p239SpE3′ were cut with Sph I and ligated before transfection. Recombinant lambda DNA and ligated plasmid DNA were transfected into cells with a DEAE-dextran procedure described previously (*6*). One rhesus monkey (316-85) was inoculated with virus produced by the lambda clone from a culture of macaque PBLs. Macaque PBLs were stimulated with phytohemagglutinin (1 μg/ml) for 2 days, transfected with DNA, and grown in the presence of interleukin-2 (IL-2). Cells were removed by centrifugation and 1 ml of the supernatant 13 days after transfection, shown to contain virus by cocultivation, was inoculated intravenously into the rhesus monkey. Four rhesus monkeys were inoculated with virus produced by the ligated plasmid clones from cultures of macaque PBLs. Supernatant containing virus 11 days after transfection was passed to a fresh macaque PBL culture and cell-free virus stock was

frozen in liquid nitrogen 7 days after infection. Before freezing, the virus stock contained 85,000 cpm per milliliter of reverse transcriptase activity measured as described previously [M. D. Daniel *et al.*, *J. Virol.* **62**, 4123 (1988)]. Portions of this stock virus (0.25 ml) were inoculated intravenously into each of the four rhesus monkeys. One rhesus monkey from this group, 452-87, has died to date. Six rhesus monkeys were inoculated with virus produced by the lambda clone from human T cell lines. Virus contained within the supernatant of HuT 78 cells after transfection was used to infect CEMx174 cells; 65 days after infection of CEMx174 cells, portions of the cell-free supernatant containing virus were frozen in liquid nitrogen. Before freezing, the cell-free supernatant contained 10^6 cpm per milliliter of reverse transcriptase activity. This stock (2 ml) was inoculated intravenously into each of the six rhesus monkeys. Three of these rhesus monkeys, 19788, 17504, and 18955, have died to date. Animals were euthanized when near death.

9. M. D. Daniel *et al.*, *J. Gen. Virol.* **68**, 3183 (1987).
10. N. W. King *et al.*, *J. Med. Primatol.*, in press.
11. D. J. Ringler *et al.*, *ibid.* **18**, 217 (1989); D. J. Ringler *et al.*, *Lab. Invest.*, in press.
12. D. J. Ringler *et al.*, *Am. J. Pathol.* **134**, 373 (1989).
13. M. S. Wyand *et al.*, *ibid.*, p. 385; M. S. Wyand *et al.*, *J. Med. Primatol.* **18**, 209 (1989).
14. D. J. Ringler *et al.*, *Ann. Neurol.* **23**, S101 (1988); G. Baskin, M. Murphey-Corb, L. Martin, *Lab Invest.* **60**, 7A (1989); K. Rappersberger *et al.*, *Intervirology* **29**, 185 (1988); E. Tschachler *et al.*, *J. Invest. Dermatol.* **88**, 233 (1987).
15. M. D. Daniel *et al.*, unpublished results.
16. M. Murphey-Corb, L. Martin, B. Davison-Fairburn, *Nonhuman Primate Models for AIDS*, abstr. 79, 93 (1989).
17. V. Hirsch, N. Riedel, J. I. Mullins, *Cell* **49**, 307 (1987).
18. G. Franchini *et al.*, *Nature* **328**, 539 (1987).
19. We thank D. Schmidt, C. Troup, D. Silva, D. Walsh, L. Antipa, J. Higgins, S. Joye, and B. Lohman for technical assistance; N. Lerche and J. Yee for serological screening at CRPRC; P. Marx for helpful discussions; and B. Blake for assistance with the manuscript. This work was supported by Public Health Service grants RR00168, RR00169, AI25328, AI62559, AI25644, and AI24292; investigator award R88D041 from the state of California Task Force on AIDS; PHS SERCA award RR0039; and a Charles A. King trust fellowship from the Medical Foundation Incorporated of Massachusetts.

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HSP104 Required for Induced Thermotolerance

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A heat shock protein gene, *HSP104*, was isolated from *Saccharomyces cerevisiae* and a deletion mutation was introduced into yeast cells. Mutant cells grew at the same rate as wild-type cells and died at the same rate when exposed directly to high temperatures. However, when given a mild pre-heat treatment, the mutant cells did not acquire tolerance to heat, as did wild-type cells. Transformation with the wild-type gene rescued the defect of mutant cells. The results demonstrate that a particular heat shock protein plays a critical role in cell survival at extreme temperatures.

CERTAIN TYPES OF STRESS, INCLUDing mild heat, condition cells and organisms to survive severe heat treatments that would otherwise be lethal. This phenomenon is called induced thermotolerance. Since virtually all treatments that induce thermotolerance also induce synthesis of heat shock proteins (hsps), it is commonly assumed that these proteins participate in protecting cells from extreme temperatures. Although accumulated circumstantial evidence supports this assumption, contradictions exist. In certain cases, thermotolerance develops in the absence of hsp synthesis, while in others, synthesis of hsps is insufficient to produce thermotolerance (*1*).

Techniques allowing production of mutations by site-directed mutagenesis in yeast have enhanced our understanding of the functions of hsps. For example, yeast cells carrying homozygous mutations in both the *HSC82* and *HSP82* genes do not grow at any temperature, while cells homozygous for mutations in either of the two genes alone can grow only at temperatures below 37°C (*2*). These proteins are apparently required for growth at all temperatures, but are required in higher concentrations for growth at higher temperatures. Cells carrying mutations in two members of the yeast *HSP70* gene family, *SSA1* and *SSA2*, are temperature-sensitive for growth at 37°C (*3*), while triple mutations in *SSA1*, *SSA2*, and *SSA4* are lethal (*4*). Cells that carry mutations in the heat-inducible polyubiquitin gene are hypersensitive to long exposure at 38.5°C, just above their maximum growth temperature (*5*).

These experiments demonstrate that certain hsps help cells to cope with temperatures at the upper end of their natural growth range or to survive long exposures to temperatures that are just beyond their growth range. However, none of these mutations compromise tolerance to extreme temperatures. In fact, the *SSA1* and *SSA2* double mutants, as well as the polyubiquitin mutant, are more tolerant than wild-type cells to extreme temperature thermotolerance. In *SSA1* and *SSA2* double mutants, cells show greater than normal expression of other hsps (*3, 6*), which might explain their increased thermotolerance. Mutations in genes unrelated to the hsp genes, such as *ard1*, *bcy1*, and $ras2^{VAL\,19}$, have been observed to block thermotolerance (*7–9*). However, these mutations appear to affect thermotolerance indirectly by blocking the entry of cells into stationary phase.

Here we report the isolation of the *HSP104* gene of yeast and the construction and analysis of strains carrying mutations in this gene. Hsp104 is the largest, heat-inducible protein in yeast cells. It is not detectable at normal growth temperatures, but becomes a major product of protein synthesis shortly after a shift to high temperatures (Fig. 1A). It is also induced during the transition to stationary phase growth and early in sporulation (*10*).

Hsp104 protein that had been purified by ion exchange chromatography and SDS–polyacrylamide gel electrophoresis was used to produce in rabbits a highly specific polyclonal antiserum (*10*). The antiserum (Fig. 1B) was used to screen an expression library of yeast genomic DNA fragments in λgt11 (*11*). Of 3×10^5 recombinant phages screened, seven gave positive signals after purification. Restriction endonuclease mapping showed that these clones share overlapping fragments. We chose to characterize the clone containing the biggest insert (3.6 kb, YS-121) (Fig. 2A).

To localize heat-induced sequences within this clone, various restriction fragments were hybridized with total RNA isolated from (i) log phase cells grown at normal temperature (25°C), (ii) log phase cells subjected to heat shock at 39°C for 30 min, and (iii) stationary phase cells grown at 25°C. Three fragments hybridized with a strongly inducible 2.7-kb mRNA in cells subjected to heat shock and stationary phase cells. The size of this RNA is in accordance with the estimated molecular size of hsp104 (104 kD). The fragments used and their relative levels of hybridization are depicted in Fig. 2B. A typical Northern blot obtained with the Bgl II–Hind III fragment is shown in Fig. 1C. The direction of transcription was determined by hybridization with RNA probes generated from the Eco RI–Hind III fragment, subcloned in both orientations into a vector that contains the T7 promoter (pVZ1) (*12*). Southern (DNA) blot analyses, conducted at both low and high stringencies, indicated that this gene is unique in the yeast genome (*13*).

To create a mutation in sequences encoding the 2.7-kb heat-inducible RNA, a 1.2-kb fragment (Apa I to Bgl II) (Fig. 2A) was

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