

Extra and intracellular expression of *Mycobacterium tuberculosis* genes

I. Smith,* O. Dussurget,[†] G. M. Rodriguez,[‡] J. Timm,* M. Gomez,* J. Dubnau,* B. Gold,[‡] R. Manganeli*

*Public Health Research Institute, New York, USA

[†]UFR de Biochimie, Université Paris, Paris, France

[‡]Department of Microbiology, New York University Medical Center, New York, USA

Summary To understand how *Mycobacterium tuberculosis* survives and grows in an infected host, we are studying the mycobacterial transcriptional machinery and its response to stresses encountered in vitro and in vivo. Much has been learned about σ factors and other transcriptional regulators concerning their roles in controlling mycobacterial gene expression. It has recently been shown that σ^A is the essential housekeeping σ factor and the alternative σ factor σ^B , not essential for growth in a laboratory setting, is required for a robust protective response to various environmental stresses. We are also studying the mechanism by which the R522H mutation in σ^A prevents the transcription of certain genes, including some that are believed necessary for virulence. Also under investigation is the mycobacterial iron acquisition apparatus and its regulation, as metabolism of this essential element plays a key role in microbial pathogenesis. We have identified and characterized the major mycobacterial iron regulator IdeR that blocks the synthesis of the iron uptake machinery and have identified target genes in *M. smegmatis* and *M. tuberculosis* that are directly repressed by IdeR. Recent studies have examined the control of *M. tuberculosis* gene expression in vivo. Among these new approaches are an in vivo expression technology system to identify *M. tuberculosis* genes that are induced in macrophages and mice and a novel RT-PCR method that allows an accurate comparison between the levels of specific mRNAs in *M. tuberculosis* grown in vitro with those found in bacteria growing in human macrophages.

INTRODUCTION

The ultimate aim of our research is to find targets that will aid in the diagnosis and/or control of *Mycobacterium tuberculosis* during the infectious process. Our strategy is to learn how *M. tuberculosis* survives and grows in the infected macrophage and host. Given the molecular genetic approach of our group, our aim was the identification and characterization of *M. tuberculosis* genes that are essential for virulence and a study of how their expression is controlled in vivo. Before this could be done, however, it was necessary to understand how mycobacterial genes are expressed outside of the host, i.e. what was the 'ground state' prior to the engulfment of *M. tuberculosis* by host macrophages. The information

obtained in these in vitro studies and the recent development of new strategies has now enabled us to identify *M. tuberculosis* genes that are induced after infection of human macrophages and mice. These initial in vitro and new in vivo approaches will be reviewed in this article that concentrates on work performed in the authors' laboratory.

SIGMA FACTOR GENETICS AND FUNCTION IN MYCOBACTERIA

Our first approach to study the basic mycobacterial apparatus was the biochemical characterization of the mycobacterial ribonucleic (RNA) polymerase and the identification of genes encoding σ factors.^{1,2} These positive transcription factors give promoter specificity to the core RNA polymerase. We initially identified and characterized *sigA* and *sigB*, closely linked genes for two highly conserved proteins of the σ^{70} class, σ^A and σ^B , respectively, from *M. smegmatis*, and did the same analysis in *M. tuber-*

Correspondence to: Issar Smith, Public Health Research Institute, 455

1st Avenue, New York, NY 10016, USA. Tel.: +212 578 0868;

Fax: +212 578 0804; E-mail: smitty@phri.nyu.edu

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culosis and *M. leprae*, in collaboration with Stewart Cole. Since that time, two other σ factors, σ^E and σ^F have been characterized in mycobacterial species.^{3,4} The former is a member of the ECF class of σ factors that are involved in stress responses, while the latter is similar to σ factors of bacilli and streptomyces that are involved in stress responses and sporulation. Analysis of the completely sequenced *M. tuberculosis* H37Rv genome has shown open reading frames that potentially encode an additional nine σ factors of the ECF class.⁵ The structures and functions of mycobacterial σ factors have been reviewed in a recent publication,⁶ and aspects of their regulation are discussed later in this article.

Two highly conserved ORFs, *orfX* and *ideR* were found closely linked to *sigA* and *sigB* in *M. smegmatis*, *M. tuberculosis* and *M. leprae* on chromosomal segments of approximately 7 Kb. PCR and Southern hybridization analyses indicated that the gene order *sigA*, *orfX*, *sigB* and *ideR* is found in all mycobacterial species thus far analyzed.² In the next part of this section, studies pertaining to *sigA* and *sigB* will be described while *ideR*, encoding a major regulator of iron uptake in mycobacteria, is discussed in the following section. *orfX* encoding a dispensible protein with six putative membrane spanning domains that has high similarity to several prokaryotic and eukaryotic transporters, will not be discussed here.

sigA

The amino acid sequences of the mycobacterial σ^A s are essentially identical, differing only in region 1.1, an amino terminal domain of variable length and sequence usually found only in major (essential, housekeeping) σ factors. This poorly conserved domain functions to prevent free σ factor from binding to promoters.⁷ The presence of an extended 1.1 region and the similarity of the mycobacterial proteins to each other and to σ^{HrdB} , the essential σ factor of *Streptomyces coelicolor*, suggested that the mycobacterial σ^A s had the same indispensable function. To confirm this hypothesis, we studied the regulation of *sigA* and attempted to inactivate it, reasoning that the gene for the major σ factor should be constitutively expressed at reasonably high levels throughout exponential growth and that it should also be indispensable.⁸ *lacZ* reporter studies, RNA measurements and immunoassays indicated that *sigA* is expressed throughout growth and early stationary phases and that σ^A levels were also constant during the growth cycle. In addition, the *sigA* promoter sequences, as measured by in vivo and in vitro primer extension analyses are highly conserved in *M. smegmatis*, *M. tuberculosis* and *M. leprae*. It was only possible to disrupt the *Msm sigA* gene by allelic replacement in a *sigA* merodiploid strain, i.e. one containing an additional *sigA* copy at the bacteriophage L5 *attB* site.

We thus conclude that σ^A is the major and essential mycobacterial σ factor.

A mutation in the *sigA* ORF, causing an arginine replacement by histidine at amino acid residue 522 attenuates the virulence of *M. bovis*.⁹ Significantly, this genetic lesion is one of the few known to affect virulence in members of the *M. tuberculosis* complex. The nucleotide sequence of the wild type *M. bovis* gene is identical to that of the *M. tuberculosis sigA*, and complementation of the attenuated *M. bovis* mutant with the *M. tuberculosis* wild type *sigA* restored virulence.⁹ The R522H mutation is adjacent to the -35 binding domain in the C-terminus of σ^A , a region that is highly conserved in major σ factors and others of the σ^{70} class and is known to interact with several positive regulators.¹⁰ This suggested to us that one or more virulence genes of *M. tuberculosis* also require a direct binding activator for their expression and that the mutation disrupts this interaction while allowing the bacteria to survive. We began a study of the phenotypic ramifications of the R522H mutation, since this analysis could provide valuable insight into *M. tuberculosis* virulence mechanisms. However, there was a possibility that suppressor mutations may have accumulated during passage of the original *M. bovis* mutant strain (ATCC 35721), potentially complicating this analysis. Therefore we wanted to create this mutation anew in a virulent *M. tuberculosis* strain. This was also attempted in *M. smegmatis* so we could study the effects of the mutation in a more tractable mycobacterial species. As discussed above, the *M. smegmatis* and *M. tuberculosis* σ^A proteins are identical, outside of region 1-1. We obtained the desired mutant in *M. smegmatis* by introducing the R522H change linked to an antibiotic resistance cassette that was inserted directly downstream of the C-terminus of σ^A . Thus, *M. smegmatis* could survive the introduction of a linked resistance cassette adjacent to the C-terminus of *sigA* along with the R522H mutation, indicating the absence of an essential downstream gene that could be disrupted by the cassette integration. However, only when a *M. tuberculosis* H37Rv strain was made diploid for *sigA* could this mutation be introduced. These results suggest that, unlike *M. smegmatis*, *M. tuberculosis* cannot survive with the R522H lesion unless suppressor mutations are also present. Since it was possible to introduce the mutation into *M. smegmatis*, we compared the phenotypes of the *M. smegmatis* wild type strain and the one containing the R522H mutation. The two strains had identical growth rates, colonial morphology and response to most stress conditions. However, the mutant was more sensitive to plumbagin (a superoxide generator), thiomethyl glycerol (a reducing agent and pleiotropic inhibitor) and the antibiotics chloramphenicol, erythromycin and tetracycline. In addition, the mutant was more resistant to gentamycin and β -lactams. We also compared, by two

dimensional gel electrophoresis, the total cell proteins produced by the wild type and mutant strains. Thus far, one clearly distinguishable protein of approximately 40 Kda is present at greatly reduced levels in the mutant. We have cloned the genetic determinant for the 40 Kda protein as well as closely linked genes and they encode polypeptides with high similarity to enzymes involved in β -oxidation of fatty acids. We are studying the regulation of the homologous proteins in *M. tuberculosis*.

sigB

The *sigB* gene encodes a 320-amino acid protein of the σ^{70} class. Phylogenetic analysis indicates it is most similar to the σ^{HrdA} of streptomycetes, a σ^{70} like protein of unknown function. *sigB* regulation in *M. smegmatis* and *M. tuberculosis* as assayed by RNA quantitation, *lacZ* reporter assays and immunoassays indicated that the *sigB* was expressed during exponential growth and did not show significantly greater expression during the stationary phase. While there is some similarity in the *sigB* promoter regions of *M. smegmatis*, *M. tuberculosis* and *M. leprae*, they are not as highly conserved as those of the *sigA* promoters from these species. We have been able to inactivate the *M. smegmatis sigB* by allelic replacement, indicating the gene is not essential (Gomez & Smith, paper in preparation). The *sigB* mutant grows normally and has wild type iron regulation, indicating that the *sigB* mutation is not polar on the downstream *ideR* gene and σ^{B} is not required for *ideR* expression (see below). However, the mutant is more sensitive to a variety of reagents, including H_2O_2 , sodium azide, plumbagin and various reducing agents but is more resistant to the front line anti-mycobacterial drug isoniazid (INH). Loss of function mutations in the mycobacterial *katG*, encoding a catalase:peroxidase, result in H_2O_2 sensitivity and INH resistance,¹¹ but activity gel assays have not shown any apparent difference between the KatG activity of wild type and *sigB* strains. The relationship between σ^{B} and oxidative stress remains unknown and is currently being studied. Also under investigation is the role of σ^{B} in *M. tuberculosis* physiology and virulence, and for these studies we are trying to inactivate *sigB* in this species.

MYCOBACTERIAL IRON REGULATION

Approximately 150–200 bp downstream of *sigB* in *M. smegmatis*, *M. tuberculosis* and *M. leprae* was found an ORF with very high similarity to DtxR, the iron binding repressor of the *tox* gene and iron uptake regulon of *Corynebacterium diphtheriae*.² The mycobacterial protein is named IdeR for iron dependent regulator. Southern and PCR analyses have shown that the *ideR* gene is found in all mycobacterial species thus far studied, and we have

recently sequenced the *M. ulcerans* gene. These experiments show that the mycobacterial IdeR proteins exhibit greater than 95% identity to each other (Dussurget & Smith, unpublished results). Primer extension analyses and immunoassays show that the *ideR* is expressed and that the protein is made in both *M. smegmatis* and *M. tuberculosis*.¹² The promoter region, located in the *sigB ideR* intergenic region, is conserved among mycobacterial species.

Our discovery of IdeR has led to a study of its role in mycobacterial iron regulation and physiology. IdeR is both a structural and functional homolog of DtxR, complementing *dtxR* loss of function mutations in *C. diphtheriae* and binding to iron regulated *C. diphtheriae* promoters at their DtxR operator sites.¹³ An *ideR* null mutation was made in *M. smegmatis* and this resulted in deregulated siderophore synthesis and an increased sensitivity to the reactive oxygen species H_2O_2 and superoxide anion.¹² In addition, the mutant had increased sensitivity to INH. The defective oxidative stress response was caused by lower levels of catalase:peroxidase (KatG) and superoxide dismutase (SodA) enzyme activities, and we have recently shown that the levels of *katG* and *sodA* protein and mRNA are lower in the mutant.¹⁴ The mechanisms by which IdeR is essential for INH resistance and a robust oxidative stress response are currently unknown and are currently under investigation.

MYCOBACTERIAL IRON/IDER CONTROLLED GENES

In collaboration with the laboratories of Randall Holmes and William Jacobs, we have shown that IdeR and iron regulate *fxbA*, encoding an enzyme involved in the biosynthesis of the *M. smegmatis* exochelin.¹⁵ IdeR directly binds to a sequence in the *fxbA* promoter region that strongly resembles the DtxR binding site.¹⁶ More recently, we have identified putative *M. tuberculosis* IdeR and iron regulated genes by in silico analysis of the Sanger and TIGR *M. tuberculosis* genomic data bases. We used the consensus DtxR/IdeR binding sequence (IdeR box) established by in vitro¹⁷ and in vivo methods¹³ to search the *M. tuberculosis* H37Rv and CSU#93 genomes, and 30 potential IdeR binding sites upstream of ORFs were observed. Some of these ORFs encoded proteins like bacterioferritin, suggesting a role in iron storage and others encoded enzymes of aromatic amino acid biosynthesis that are presumably involved in siderophore biosynthesis as in other microorganisms.¹⁸ Another group showed strong similarity to enzymes of cell wall synthesis and restructuring. In the absence of demonstrated functions, we initially named these genes *irg* (iron regulated gene), and we are now studying their regulation and function. In the first and most highly studied example, the putative

IdeR box was found between two divergently transcribed ORFs, 31 bp upstream of one (*irg-2*) and 70 bp upstream of the other (*irg-1*). *irg-2* encodes a protein of unknown function with six transmembrane domains that is a member of the PPE family of *M. tuberculosis* proteins, a large group of rarely uncharacterized *M. tuberculosis* proteins that have been annotated in the recently sequenced *M. tuberculosis* H37Rv genome. It shows the greatest similarity to an *M. leprae* protein, the 45.9 Kd antigen. *irg-1* encodes the *M. tuberculosis* *hisE*, with *hisG* directly downstream. These two genes encode enzymes functioning early in histidine synthesis. Significantly, *hisE* and *hisG* are separate from the other genes in the histidine pathway, unlike the situation in most other bacteria in which all of the histidine biosynthetic genes are linked.¹⁹ Another difference is the fact that the *hisI* and *hisE* are unlinked in *M. tuberculosis* and in other actinomycetes, while they are joined in most other bacteria and encode fused HisIE proteins.

To show that the sequence resembling an IdeR binding site was functional, we initially showed that a 200 bp deoxyribonucleic (DNA) fragment containing the putative IdeR box and the divergently transcribed *irg-1* and *irg-2* promoters was specifically retarded by the *M. tuberculosis* IdeR and that this protein bound to the IdeR box as assayed by DNase protection. The regulation of the two genes was then analyzed by constructing separate *lacZ* transcriptional fusions with their promoters in an integrational vector that were then transformed into *M. tuberculosis* H37Rv. Both genes were repressed by high iron. We do not have an *ideR* mutant in *M. tuberculosis*, but to test the effect of IdeR on the regulation of these two genes, the same reporter constructs were integrated into wild type *M. smegmatis* MC²155 and an isogenic *ideR* mutant, SM3. As in *M. tuberculosis*, the two genes were repressed by iron in MC²155. However, iron had no effect on *irg-1* and *irg-2* expression in SM3, indicating IdeR represses these genes in bacterial cells as well as binding to this region in test tube experiments (Rodriguez et al, paper in preparation). A role for iron in the regulation of histidine biosynthesis has not been described in other bacteria.¹⁹ However, this amino acid could be a precursor for a *M. tuberculosis* siderophore as is true for the exochelin MN of *M. neoaurum*.²⁰ In this case, it would not be surprising that iron would have some modulatory effects on histidine biosynthesis. Of great significance, as discussed below, is our recent observation that *irg-1* (*hisE*) is induced after *M. tuberculosis* infection of macrophages.

AN IVET SYSTEM FOR *M. TUBERCULOSIS* GENES INDUCED IN VIVO

Major aims of our work include the identification of *M. tuberculosis* genes that are induced during infection

and the elucidation of their roles in pathogenesis. Several strategies have been employed to isolate differentially expressed bacterial genes in other bacterial systems. Among these, some of which have been used in mycobacteria, are: one or two dimensional gel electrophoresis of proteins and the subsequent cloning by reverse genetics of the genes encoding proteins that show changed levels;^{21,22} RNA based methods like cDNA (subtractive hybridization)²³ and differential display cloning.²⁴ In addition, the new fixed array technology can be used in conjunction with DNA sequence information to determine the differential expression of bacterial genes in varying environments.²⁵ Genes from bacterial pathogens that are induced during infection have been isolated by in vivo expression technology (IVET)²⁶ and by green fluorescent protein reporter activity coupled to FACS cell sorting,²⁷ while genes essential for survival in the infected host have been identified by negative selection with signature tagged mutagenesis.²⁸

An IVET system to isolate *M. tuberculosis* genes whose expression is induced in macrophages and in animals has been developed in our laboratory. The method utilizes the ability of the mycobacterial InhA protein to cause resistance to INH when it is overproduced.²⁹ InhA is an enoyl-reductase involved in mycolic acid biosynthesis and mutations in *inhA* cause INH resistance by preventing or reducing the binding of the drug to the protein. InhA overproducing mycobacterial strains are resistant to INH, presumably by sequestration of the antibiotic. Since wild type *M. tuberculosis* strains are quite sensitive to INH both in vivo and in vitro, we expected that the use of INH resistance in an IVET system would give a very clean, efficient selection for differentially expressed genes.

A mycobacterial/*Escherichia coli* shuttle vector (pJD32) containing a promoterless *M. smegmatis* *inhA* gene was constructed and to first demonstrate the feasibility of the INH-IVET system, the *M. bovis* bacille Calmette-Guérin *hisp60* promoter was cloned upstream of *inhA* in pJD32. This new construct, pJD33, conferred high level resistance to INH when transformed into *M. tuberculosis* H37Rv as measured either in differentiated THP-1 macrophages or on plates, while bacteria with the vector alone showed the normally low INH resistance of *M. tuberculosis*. *M. tuberculosis* carrying pJD33 also survived three log orders better in mice treated with INH for 5 weeks than bacteria containing the vector alone, as measured by bacillary load. Having demonstrated that the method worked, a library of *M. tuberculosis* H37Rv chromosomal fragments of 200–500 bp was then cloned into vector pJD32. These plasmids were then transformed into *M. tuberculosis* H37Rv and pools of the resulting bacteria were used for different types of in vivo selections in the presence of INH. One type of selection used several passages in human alveolar macrophages, another used differentiated

THP-1 cell lines, and the last method selected for bacteria containing the plasmid library that survived in mice that have been treated with INH. In each case we were selecting for *M. tuberculosis* strains that survived in vivo passage in the presence of INH (0.5 µg/ml) but showed wild type *M. tuberculosis* INH resistance in vitro (LD 50 of 0.05 µg/ml). Putative clones were repassaged in vivo at least once. We have isolated several clones from each selection method, but one, *ive-4*, originally selected by two passages in human alveolar macrophages, has been studied in greatest detail. The DNA sequence of *ive-4* was then obtained, and the corresponding ORF was identified by screening the Sanger and TIGR *M. tuberculosis* genomic data bases. The ORF has very high homology to MoaB, an enzyme involved in the molybdopterin biosynthetic pathway. Molybdopterin is an essential cofactor for many enzymes that function during anaerobic conditions, notably the respiratory nitrate reductase.³⁰ Our result was very suggestive since respiratory nitrate reductase levels increase early in the development of persistence in an in vitro model of *M. tuberculosis* latency³¹ (Wayne, personal communication).

QUANTITATIVE ANALYSIS OF IN VIVO GENE EXPRESSION

To accurately compare the in vivo and in vitro expression of genes we had identified by the IVET strategy and other genes of interest, we developed a real time RT-PCR method based on molecular beacon fluorescence. Molecular beacons are sequence specific oligonucleotides that fluoresce when annealed to complementary DNA strands. These interactions can be combined with PCR amplification of the target sequence to measure input DNA levels by real time fluorescence.³² We have combined this technique with a standard RT-PCR protocol so that the levels of specific mRNAs could be sensitively and accurately measured in *M. tuberculosis* strains grown in different conditions, including macrophages and granulomas. In these experiments, total RNA is purified from *M. tuberculosis*, copied by reverse transcriptase, using gene specific primers, and the resulting cDNA is amplified in the presence of a molecular beacon that is complementary to a region within the amplicon. The method is extremely sensitive, requiring the equivalent of 10 ng of RNA per RT-PCR reaction and is accurate as there is a linear response between amounts of RNA and the fluorescent output over a range of 10² to 10⁵ molecules of nucleic acid.

To test this new RT-PCR system, before we analyzed in vivo expression of *M. tuberculosis* genes, we studied the in vitro regulation of several *M. tuberculosis* σ factor genes. *M. tuberculosis* can encounter different environments during the course of an infection, both in the acute and chronic phases of the disease and alternative σ factors are

known to provide to bacteria a means of regulating gene expression in response to changing environments. There are also many examples of bacterial virulence determinants whose expression is regulated by alternative σ factors. Thus we believed that information gathered from these in vitro studies of σ gene expression would be useful for an understanding of *M. tuberculosis* virulence. *M. tuberculosis* H37 RV cultures were exposed to a variety of environmental stresses, including exposure to anionic detergents, H₂O₂, heat shock, cold shock and low aeration. Molecular beacon RT-PCR analysis was then used to measure mRNA levels of the previously mentioned *sigA*, *sigB*, *sigE*, *sigF* and six of the nine newly described ECF σ factor genes, i.e., *sigC*, *sigD*, *sigG*, *sigH*, *sigI* and *sigM*. (Manganelli et al paper in preparation). Transcripts from all of the σ factor genes were detected during exponential growth, indicating that they are all expressed. To briefly highlight the most significant responses, *sigB* mRNA levels increased 10-fold after detergent treatment and 20-fold after heat shock while the same stresses caused six-fold and three-fold increases in *sigE* transcripts. *sigH* transcripts increased 20-fold after heat shock. We are currently studying the levels of these mRNAs during *M. tuberculosis* infection of macrophages, as described below.

Having tested the utility of the molecular beacon RT-PCR method with in vitro cultures of *M. tuberculosis*, as described above, we have begun similar studies with *M. tuberculosis* H37Rv grown in the human monocytic cell line THP-1. In our early experiments, we prepared 20–40 µg of RNA from *M. tuberculosis* growing in large THP-1 cultures that were induced to differentiate with forbol ester. These relatively large amounts of RNA were useful in working out conditions of RNA extraction from macrophages and doing various controls. Since then we have been able to scale down our methods so that sufficient *M. tuberculosis* mRNA for a limited number of RT-PCR assays can be prepared from 1.0 ml *M. tuberculosis* infected THP-1 cultures.

Iron uptake is important for all living organisms and is required by pathogens during infection, but little is currently known about the role of bacterial iron acquisition during *M. tuberculosis* infections and whether iron is limiting in the mycobacterial phagosome. To begin a study of this important area, we are measuring the expression of *M. tuberculosis* iron regulated genes in macrophages. *M. tuberculosis* H37Rv cells, grown in vitro in Middlebrook 7H9 medium that contains levels of iron that repress iron regulated genes (187 µM), were used to infect differentiated THP-1 cells. Bacteria were isolated 5 h and 3 days after infection and mRNA levels of the iron repressible *irg1* (*hisE*) gene were measured, using the molecular beacon RT-PCR method. *irg1* RNA levels increased four-fold 5 h after infection and 10-fold after 3 days, compared to its levels in vitro.

FUTURE PROSPECTS

We are continuing our search for genes that require a wild type σ^A for their expression, as this is expected to lead to the identification of a positive regulator for virulence gene expression. We have also developed methods for isolating *M. tuberculosis* genes that are induced during macrophage and mouse infection and for accurately measuring their in vivo expression. We will now investigate the role of these genes in virulence by inactivating them and then measuring the ability of mutants to survive and grow in macrophages and mice. The observation that the iron regulated *irg-1* (*hisE*) is induced 5–10-fold in macrophages strongly suggests that the milieu of the mycobacterial phagosome is limiting for iron. By inactivating some of the iron regulated genes we have putatively identified in the *M. tuberculosis* genome, we expect to disrupt the bacterium's iron acquisition machinery. *M. tuberculosis* strains with these mutations should be attenuated for survival and virulence in animal hosts. Lastly, we have now shown that several genes encoding alternative σ factors, i.e. σ^B and some of the ECF class σ factors, are also induced in different stress conditions. By learning how these σ factor genes are regulated in macrophages and which genes require these σ factors for their expression, we will be able to identify several new potential targets for tuberculosis diagnosis and therapy. These experiments are currently in progress.

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