disease, it is reasonable to propose that an excess gene dosage of certain genes, with overproduction of the corresponding proteins (*11*) could cause these changes to occur prematurely. Moreover, even if Alzheimer's disease requires a duplication of a segment of chromosome 21 containing the AD-AP gene, it does not follow that all bearers of this duplication will develop the disease. The roles played by faulty regulatory mechanisms and environmental stress are still poorly understood, but are certainly important.

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Transposition of Gram-Positive Transposon Tn*916* in *Acholeplasma laidlawii* and *Mycoplasma pulmonis*

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Mycoplasma genetics has been limited by a lack of genetic tools such as selectable markers, methods to transfer DNA, and suitable vectors for cloning. Studies were undertaken to examine the potential of using the streptococcal transposon Tn*916* as a mycoplasma genetic tool. The *Escherichia coli* plasmid pAM120, which contains Tn*916*, was transformed into *Acholeplasma laidlawii* and *Mycoplasma pulmonis*. Transposition of Tn*916* into the mycoplasma chromosome apparently occurred by an excision-insertion mechanism. This example shows that newly introduced DNA from other bacteria can be successfully expressed in mycoplasma and that Tn*916* should serve as a powerful genetic tool for the study of mycoplasmas.

MYCOPLASMAS ARE WALL-LESS prokaryotes that cause disease in plants, insects, and animals, including man. The five currently recognized genera of mycoplasmas, *Acholeplasma, Spiroplasma, Mycoplasma, Ureaplasma,* and *Anaeroplasma*, are a diverse group of related organisms that have evolved from Gram-positive bacteria (*1*). The development of mycoplasma genetics has been hampered by a lack of selectable markers and a lack of methods to transfer DNA such as conjugation, transformation, and transduction. Therefore, most studies in mycoplasma genetics have been limited to the cloning and subsequent analysis of mycoplasma DNA in *Escherichia coli*; few studies in mycoplasma genetics have involved the isolation and analysis of mutants. In this report we describe the transformation of mycoplasmas with the transposable element Tn*916*. The convenience of insertional mutagenesis through transposition of Tn*916* should stimulate the field of mycoplasma genetics.

Tetracycline-resistant isolates of *Mycoplasma hominis* and *Ureaplasma urealyticum* have DNA sequences homologous to the streptococcal *tetM* determinant (*2, 3*). The *tetM* determinant may have disseminated from streptococci via a conjugative transposon—for example, Tn*916*, a broad–host range transposon with which *tetM* is associated (*4–8*). The plasmid pAM120 (21 kilobases) contains Tn*916* (16 kilobases) cloned into the vector pGL101 in *E. coli* strain DH1 (*8*). We used pAM120 to transform mycoplasmas to determine whether Tn*916* will function in these cells. We were successful in transformation of both *Acholeplasma laidlawii* and the murine pathogen *Mycoplasma pulmonis*.

Transformations were based on the polyethylene glycol (PEG) procedure developed for transfection of *A. laidlawii* with mycoplasma viral DNA (*9*). *Acholeplasma laidlawii* cells were propagated in tryptose broth (*10*) and assayed as colony-forming units (CFU) on mycoplasma agar plates (*11*). Ten micrograms of pAM120 DNA were used to transform 250 μl (about 2×10^7 CFU) of cells from a culture in logarithmic growth phase. After transformation, cells were incubated at 37°C in tryptose broth for 30 minutes to allow for expression of the tetracycline-resistance determinant and then plated onto freshly made agar plates supplemented with tetracycline at a concentration of 2 μg/ml. Selection with tetracycline at a concentration of 4μg/ml gave somewhat more inconsistent transformation frequencies, and incubation for more than 30 minutes before plating did not increase transformation frequencies. The transformation mixtures were assayed on agar plates in incubation at 37°C in the dark (tetracycline is light-sensitive) for 4 days. Colonies were picked by stabbing the plate with a sterile 1-ml pipette and placing the agar plug in 1 ml of tryptose broth. This culture was incubated at 37°C for 2 hours; tetracycline was added to a concentration of 4 μg/ml; and the culture was incubated again for 2 to 3 days until turbidity was observed. If tetracycline was added immediately after picking colonies, some of the cultures did not grow. Waiting 2 hours before adding the tetracycline alleviated this problem (*12*). Once the isolates were actively growing in liquid medium, they could be incubated in medium containing high levels of tetracycline, at least 40 μg/ml, and still retain cell growth.

Transformation of *M. pulmonis* was similar to that of *A. laidlawii*. Mycoplasma medium (*11*), instead of tryptose broth, was used for propagation of *M. pulmonis*. Important for obtaining an efficient protocol for transformation of *M. pulmonis* was the realization that mycoplasma medium and tryptose broth were inhibitory. The first step in the protocol for transformation of *A. laidlawii* is to harvest cells by low-speed centrifugation and resuspend the cell pellets in fresh tryptose broth (*9*). We removed the inhibitory medium from *M. pulmonis* by resuspending the cell pellets in buffer. Examination of various buffers showed that the optimum PEG concentration was dependent on the buffer (*13*). After transformation of *M. pulmonis*, the incubation time allowed for expression of the *tetM* determinant was 2 hours.

Acholeplasma laidlawii was chosen as the recipient for our initial transformation studies because a PEG-mediated transformation procedure had already been developed for transfection of this mycoplasma with viral DNAs. Strain 8195 was chosen because it apparently lacks a restriction and modification system (*14*). We obtained tetracycline-resistant transformants of *A. laidlawii* strain

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8195 at a frequency of about 10^{-6} per CFU. We extended our findings by successfully transforming *A. laidlawii* strain JA1 (*15*), a restriction- and modification-positive strain from which strain 8195 was derived. Transformation of strain JA1 with pAM120 also occurred at a frequency of 10^{-6} per CFU. Evidently, the JA1 restriction system was not a factor in these experiments; it is thought to be based solely on restriction of DNA containing 5-methylcytosine (*14*). Transformation of *M. pulmonis* strain UAB 6510 (*16*) occurred at a frequency of about 5×10^{-6} per CFU, with cells suspended in phosphate-buffered saline and PEG concentration of 36% (*13*). Spontaneous mutants resistant to tetracycline were not detected in control transformations of *A. laidlawii* and *M. pulmonis* that did not receive pAM120. The spontaneous mutation rate for tetracycline resistance in these mycoplasmas is less than 10^{-9}.

The tetracycline-resistant clones were not contaminants. To compare the DNA of the transformants with DNA of cells that were not transformed, we analyzed restriction endonuclease–digested material on agarose gels stained with ethidium bromide. All isolates had essentially identical banding patterns. We also confirmed the identity of the isolates by examining mycoplasma virus susceptibility with a plaque-forming-unit assay (*17*). Tetracycline-resistant transformants of *A. laidlawii* retained susceptibility to mycoplasma virus L3 (*18*), and tetracycline-resistant *M. pulmonis* isolates retained susceptibility to the virus P1, which is specific for *M. pulmonis* (*19*).

DNAs from the tetracycline-resistant transformants were analyzed on Southern blots probed with pAM120. Sequences homologous to pAM120 were detected as part of the mycoplasma chromosome (Fig. 1); no pAM120 sequences were detected as circular extrachromosomal DNA (lane 1 in Fig. 1, A and B). There was no DNA homology between pAM120 and either *A. laidlawii* (lane 7 in Fig. 1A) or *M. pulmonis* cells that were not transformed. Restriction endonuclease Hind III cleaved pAM120 at a single site (lane 8 in Fig. 1A). Digestion of the transformed mycoplasma DNAs with Hind III resulted in two bands with homology to pAM120 (lanes 2 to 6 in Fig. 1A and lane 2 in Fig. 1B). No two transformants of *A. laidlawii* had the same Hind III banding pattern, showing the pAM120 sequences were inserted at a different site in each isolate.

When propagated in *E. coli* strain DH1 in the absence of tetracycline, pAM120 spontaneously loses its Tn*916* sequences, leaving a 5.1-kb plasmid designated pAM120LT, which is ampicillin-resistant but tetracycline-sensitive (*4*, *8*). We isolated pAM120LT and used it as a hybridization probe to analyze the transformants of *A. laidlawii* on Southern blots similar to those shown in Fig. 1. Plasmid pAM120LT retained homology with pAM120 but lacked homology to DNA from the tetracycline-resistant transformants. These data indicate that Tn*916* was excised from pAM120 and inserted into the chromosome of the transformed mycoplasma cells. Since Tn*916* transposition apparently occurs through an excision-insertion mechanism in streptococci (*6*, *8*), it appears that properties of Tn*916* in mycoplasmas are the same as they are in streptococci.

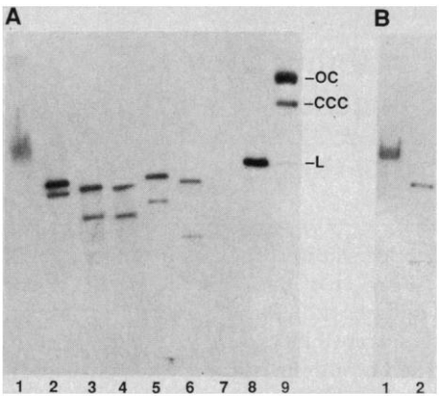

Fig. 1. Autoradiogram of Southern blots probed with pAM120 DNA. Mycoplasma DNA (5 μg) or plasmid DNA (0.05 μg) were loaded onto 0.6% agarose gels. After electrophoresis, DNA was blotted onto nitrocellulose filter paper and hybridized to ^{32}P-labeled pAM120. pAM120 was labeled by nick translation to a specific activity of 10^8 counts per minute per microgram of DNA. Plasmid DNA was isolated from *E. coli* strain DH1 cells by the cleared lysate method (*21*). Mycoplasma chromosomal DNA was isolated by phenolchloroform extraction of SDS-lysed cells (*22*). Agarose gel electrophoresis, Southern blotting, nick translation, and hybridization conditions were as described elsewhere (*21*, *23*). (**A**) Analysis of *A. laidlawii* strain 8195 DNAs. Each lane of the gel was loaded with the following DNAs: (lanes 2 to 6) DNA from five independent, tetracycline-resistant transformants of strain 8195, digested with Hind III; (lane 1) same DNA as in lane 6, but undigested; (lane 7) DNA from strain 8195, which was not transformed; (lanes 8 and 9) pAM120 DNA, digested with Hind III and undigested, respectively. (**B**) Analysis of *M. pulmonis* DNAs: (lanes 1 and 2) DNA from a tetracycline-resistant transformant of *M. pulmonis* strain UAB 6510, undigested and digested with Hind III, respectively. Restriction endonucleases were used according to the directions of the supplier (Bethesda Research Laboratories, Gaithersburg, Maryland). Digestion of chromosomal DNA with ribonuclease A (Sigma) (20 μg/ml) was simultaneous with restriction endonuclease digestion to degrade contaminating RNA. The circular and linear forms of pAM120 DNA are labeled as open circular (OC), covalently closed circular (CCC), and linear (L). The identity of these circular forms was confirmed by mild S1 nuclease digestion which converted the CCC DNA to OC DNA and finally to L DNA.

The tetracycline-resistant transformants of *A. laidlawii* were stable. We passaged the clones by daily transfer from an actively growing culture into fresh medium, each transfer being a 1:100 cell dilution. DNA was analyzed on Southern blots as described above, and no change was detected in the Hind III banding pattern of the DNA with up to 14 passages. The presence or absence of tetracycline during passaging had no effect on this stability.

Tn*916* will be a useful tool for the study of mycoplasma genetics via insertional mutagenesis. Since Tn*916* functions in *A. laidlawii* and *M. pulmonis*, and since sequences homologous to *tetM* have been found in *M. hominis* and *U. urealyticum*, it seems likely that Tn*916* will function in mycoplasmas in general. The properties of Tn*916* that make it useful in the genetic analysis of Gram-positive bacteria have been discussed elsewhere (*8*). Briefly, the insertion of Tn*916* via transposition should be a convenient form of mutagenesis. Once mutants are identified, the mutated genes can be isolated by cloning the inserted Tn*916* along with its flanking sequences. Once cloned in an *E. coli* vector, Tn*916* can be precisely excised by propagation of the cells in the absence of tetracycline and thereby regenerate the gene that had been mutated. The stability of the inserted Tn*916* sequences indicates that the mutants generated by Tn*916* transposition will be sufficiently stable to allow for screening by the use of functional assays.

The data presented here indicate that both the *tetM* determinant and the Tn*916* genes required for transposition are expressed in mycoplasmas. This suggests that expression of genes from other systems may occur readily in mycoplasmas. If so, the failure of investigators to find cloning vectors that function in mycoplasmas may reflect difficulties in DNA replication rather than a lack of expression of antibiotic resistance determinants. This suggests that it may be necessary to construct a mycoplasma cloning vector by combining an antibiotic resistance determinant, such as *tetM*, with a mycoplasma replicon, such as an origin of replication from either one of the mycoplasma viruses or one of the plasmids found in species of *Spiroplasma* (*20*).

Another aspect of Tn*916* that should be useful for the development of mycoplasma genetic techniques is the transposon's conjugative properties. The fertility factors necessary for conjugation of Tn*916* are thought to be self-encoded (*4*). Therefore, it is possible that Tn*916* will be conjugative in mycoplasmas, and if so, Tn*916* can be used to develop efficient procedures for mycoplasma mating.

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Human Retinoblastoma Susceptibility Gene: Cloning, Identification, and Sequence

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Recent evidence indicates the existence of a genetic locus in chromosome region 13q14 that confers susceptibility to retinoblastoma, a cancer of the eye in children. A gene encoding a messenger RNA (mRNA) of 4.6 kilobases (kb), located in the proximity of esterase D, was identified as the retinoblastoma susceptibility (RB) gene on the basis of chromosomal location, homozygous deletion, and tumor-specific alterations in expression. Transcription of this gene was abnormal in six of six retinoblastomas examined: in two tumors, RB mRNA was not detectable, while four others expressed variable quantities of RB mRNA with decreased molecular size of about 4.0 kb. In contrast, full-length RB mRNA was present in human fetal retina and placenta, and in other tumors such as neuroblastoma and medulloblastoma. DNA from retinoblastoma cells had a homozygous gene deletion in one case and hemizygous deletion in another case, while the remainder were not grossly different from normal human control DNA. The gene contains at least 12 exons distributed in a region of over 100 kb. Sequence analysis of complementary DNA clones yielded a single long open reading frame that could encode a hypothetical protein of 816 amino acids. A computer-assisted search of a protein sequence database revealed no closely related proteins. Features of the predicted amino acid sequence include potential metal-binding domains similar to those found in nucleic acid–binding proteins. These results provide a framework for further study of recessive genetic mechanisms in human cancers.

RETINOBLASTOMA, THE MOST COMMON intraocular cancer of childhood, occurs in both hereditary and nonhereditary forms. The former is characterized by early age of onset and multiple tumor foci compared to the nonhereditary type, which occurs later with a single, unilateral tumor (*1*). Susceptibility to hereditary retinoblastoma is transmissible to offspring as an autosomal-dominant trait with 90% penetrance, and the tumor is a prototypic model for the study of genetic determination in cancer (*2*).

The genetic locus determining retinoblastoma susceptibility was assigned to band q14 of chromosome 13, along with the gene for the polymorphic marker enzyme esterase D, by examination of cytogenetic deletions (*3*). The close linkage of these loci was confirmed by studies of retinoblastoma pedigrees (*4*). The retinoblastoma susceptibility locus was further implicated in nonhereditary retinoblastoma by observations of frequent chromosome 13 abnormalities in tumor karyotypes and reduced esterase D activity in tumors (*5*). Benedict *et al.* proposed that inactivation of both alleles of a gene (RB) located in region 13q14 resulted in retinoblastoma, based in part on a case of hereditary retinoblastoma in which both RB alleles were inferred to be absent (*6*). Recently an assumption made in this case has been disproved (*7*), namely that the absence of esterase D activity implied loss of both esterase D and RB genes. Nonetheless, Cavenee *et al.* (*8*) found that chromosome 13 markers that were heterozygous in somatic cells often became homozygous or hemizygous in retinoblastoma tumors, and Dryja *et al.* (*9*) demonstrated homozygous 13q14 deletions at the molecular level in 2 out of 37 retinoblastoma tumors. These experiments provided evidence that the proposed RB gene indeed functions in a "recessive" manner at the cellular level (*1, 10*), in distinction to the "dominant" activities of classical oncogenes (*10, 11*) as measured, for example, by transfection assays.

Given the importance of RB as a novel type of cancer gene, we undertook to isolate this gene by "chromosome walking" from other chromosome 13 markers. Since nothing was known of RB gene expression or products, candidate genes would be identified on the basis of appropriate chromosomal location and presumed "recessive" behavior: that is, an intact RB gene should be expressed in retinal tissue but not in retinoblastomas. Our initial starting point was the gene encoding esterase D (*12*), which is linked to the retinoblastoma susceptibility locus in band 13q14.11 (*4, 13*) within an estimated 1500-kilobase range (*14*). The esterase D complementary DNA (cDNA) clone EL-22 was used as a probe to isolate its genomic DNA clones. Distal DNA segments of these genomic clones were used in turn to isolate additional genomic clones. At 20-kb intervals in walking regions, unique sequences were identified that were used as probes to isolate cDNA clones from fetal retina and placenta libraries. By alternately screening genomic and cDNA libraries, we have established a bidirectional chromosome walk covering 120 kb (Fig. 1). Two cDNA clones, SD-1 and SD-2, were isolated by means of probes 5′ to the esterase D gene. Chromosome walking 3′ to the esterase D gene was hampered by a 20-kb region

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