Absence of Mycoplasmal Gene in Malignant Mammalian Cells Transformed by Chronic Persistent Infection of Mycoplasmas (44271)

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Abstract. **Chronic persistent infections by mycoplasmas induced malignant transformation of C3H mouse embryo cells that normally had never been reported to undergo spontaneous transformation. This mycoplasma-mediated oncogenic process had a long latency (more than 7 weeks of continuous mycoplasmal infection) and showed a multistage progression characterized by reversibility (at least up to 11 weeks of mycoplasmal infection) and irreversibility of malignant properties upon removal of the mycoplasma from culture. Further prolonged infections (18 weeks) by *Mycoplasma fermentans* or *M. penetrans* resulted in permanent transformation of these C3H cells that no longer required the continued presence of the transformation-inducing mycoplasmas in cultures to retain their malignant properties. Previous studies of viral oncogenesis revealed that virus-transformed cells always had viral gene(s) present. Integration of viral gene(s) apparently played an important role in the process of oncogenesis. In this study, we examined if the continued presence of any mycoplasmal gene(s) in mammalian cells, in whatever form, was also crucial in causing malignant cell transformation. Representational difference analysis (RDA) was a recently developed powerful technique to compare differences between two complex genomes. In the RDA system, subtractive and kinetic enrichment was used to purify and isolate restriction endonuclease gene fragment(s) of mycoplasmal origin, presumably present only in mycoplasma-transformed C3H cells, but not in nonmycoplasma-exposed control C3H cells. After three rounds of subtractive hybridization following PCR enrichment for each of three different restriction enzymes DNA digests, no gene fragment of mycoplasmal origin was amplified or identified in the permanently transformed C3H cells. Differing from tumorigenesis in animal cells induced by most oncogenic viruses or in plant cells induced by Agrobacteria, mycoplasmas evidently did not cause malignant transformation by integrating their gene(s) into the mammalian cell genome.** [P.S.E.B.M. 1998, Vol 218]

Malignant transformation of mouse embryonic cells (C3H/10T1/2) induced by prolonged persistent infection of mycoplasmas was recently reported (1). In contrast to most virus-transformation models, the mycoplasma-mediated oncogenic model required a long latency and showed a gradual progression of developing malignant properties in the infected mammalian cells. A subsequent study revealed that high level expressions of H-*ras* (7–16-fold increase) and c-*myc* (5–8-fold increase) oncogenes were closely associated with the manifestation of malignant characteristics such as morphological changes and uncontrolled cell growth in mycoplasma-infected C3H cells. The marked expression of H-*ras* or c-*myc* gene in C3H cells depended on the continued presence (at least up to the 11th week) of the mycoplasma in culture (2). Upon eradication of the transformation-inducing mycoplasma from culture, H-*ras* and c-*myc* mRNA rapidly declined to the undetectable level of nontransformed parental C3H

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cells, and all malignant properties of the once-fully-transformed C3H cells quickly reversed.

However, following 18 weeks of prolonged mycoplasmal infection, constitutive expression of H-*ras* and c-*myc* genes at a high level was established in C3H cells. These C3H cells exhibited prominent chromosomal changes, became irreversibly transformed and no longer required the continual presence of mycoplasmas in culture (1). They permanently retained all the malignant properties including the ability to form tumors in animals even though the transformation-inducing mycoplasma was eradicated from culture. In oncogenesis associated with viral infections, integration of viral genes into the mammalian cell genome plays an important role in causing malignant cell transformation, and the viral gene's continued presence is necessary for maintenance of the malignant state (3–7). Thus, the transforming virus' genome is continuously present in cancer cells. Moreover, a horizontal virulence gene transfer from Agrobacterium to a variety of higher plants has been shown to play a key role in inducing tumorous root proliferation or crown gall tumors (8).

In an attempt to understand the possible mechanisms associated with mycoplasma-mediated cell transformation, we examined the permanently transformed C3H cells for presence of mycoplasmal gene(s) after the mycoplasmas were eradicated from culture by antibiotic treatment. We detected no mycoplasmal gene by blot hybridization using the entire mycoplasmal genome or cloned mycoplasmal genes such as psb-8.6, psb-2.2, M6A1-2.9 or M6A1-3.1 (9, 10) as probes. However, we wondered if a fragment(s) or short sequence of mycoplasmal DNA not detectable by blot hybridization, could have inserted itself into the cellular DNA. Insertion of mycoplasmal DNAs in strategic locations conceivably could promote expression of certain oncogenes in the transformed cells.

Recently, a new system using subtractive hybridization and PCR amplification (SHPCRA) called representational difference analysis (RDA) was reported (11). RDA can amplify and clone the difference of DNA between two complex genomes with high sensitivity. In the RDA system, two populations of DNAs that are suspected to differ are generally referred to as the tester DNA and the driver DNA. The tester DNA may contain a target sequence that is not present in the driver DNA. DNAs or genes that are present only in the tester will be exponentially enriched by PCR amplification after each round of subtractive hybridization using an excessive amount of driver DNA. This highly sensitive technique has proved to be extremely powerful in finding hidden infectious agents associated with various diseases and gene rearrangement during carcinogenesis (12, 13).

DNAs from C3H cells irreversibly transformed by prolonged infections of *Mycoplasma fermentans* and *M. penetrans* that were subsequently eradicated from culture using antibiotics (C3H/Mf/P18-Cip and C3H/Mp/P18-Cip) (1) were used as tester DNAs and DNA from parental control C3H cells as driver DNA. Three restriction endonucleases *Bam* HI, *Xba* I, and *Hind* III were used to prepare representations of the tester and the driver DNAs. In order to verify the technical efficacy of our RDA study, we set up model testers by spiking the driver C3H cells' DNA with various fragments of *M. fermentans* DNA and testing in parallel. Using the RDA technique, after three consecutive rounds of subtractive hybridization and PCR amplification of nonhybridized DNA for each of the three restriction enzyme digests, we did not detect any DNA of possible mycoplasmal origin in the permanently transformed C3H cells.

Materials and Methods

Cell Culture. C3H, C3H/10T1/2, permanently transformed C3H cells induced by 18 weeks of persistent *Mycoplasma fermentans* (C3H/Mf/P18-Cip) and *M. penetrans* (C3H/Mp/P18-Cip) infections as well as the cell culture techniques were previously described in detail (1). Mycoplasmas were eradicated from cultures of C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells by three cycles (one cycle/week) of ciprofloxacin (Cip) (Sigma Chemical Co., St. Louis, MO) treatment followed by PCR verification (1). These irreversibly transformed cells retained all of their malignant characteristics without the continued presence of mycoplasmas.

Preparation of Genomic DNA for Driver, Tester, and Model Testers. Genomic DNA was isolated from C3H, C3H/Mf/P18-Cip, and C3H/Mp/P18-Cip cells by phenol extraction followed by phenol chloroform extraction as described previously (14). DNA from C3H cells was used as the driver, and DNAs from C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells were the testers. Model testers, positive controls for the technique, were prepared by spiking i) 25 pg of genomic DNA from *M. fermentans* (*incognitus* strain); or ii) 0.25 pg of cloned 2.2-Kb DNA (plasmid psb 2.2-Kb) of *M. incognitus* (15) into 1 μg of driver DNA.

Adaptor. Oligonucleotides used for RDA adaptors (or primers) were synthesized using a Perkin-Elmer/ABI DNA synthesizer (Model 392/394 DNA/RNA Synthesizer), heat deblocked and Sephadex G25 column purified (Pharmacia Biotech, Piscataway, NJ). The sequences of the adaptors for each restriction endonuclease used in this study are shown in Table I. Some of the adaptor sequences were originally described by Lisitsyn *et al.* (11).

Preparation of Amplicons. The amplicons were prepared according to procedures previously described in detail (11). Briefly, genomic DNA for driver or tester (including model tester) was separately digested by restriction endonucleases, *Bam* HI, *Xba* I, or *Hind* III. One μg of each digested DNA was mixed with 0.5 nmol of 24-bp and of 12-bp unphosphorylated oligonucleotide (Table I, primer set 1) in 30 μl of T4 DNA ligase buffer. The oligonucleotide was then ligated to digested DNA fragments by overnight incubation with 400 U of T4 DNA ligase (New England Biolabs, Inc., Beverly, MA) at 16°C. After ligation, both tester and driver DNA were amplified for 20 cycles (each cycle including 1 min incubation at 95°C, 3 min at 72°C).

Table I. DNA Sequences of Adaptors Used for Representational Difference Analysis

Restriction enzymes	Primer set	Name	Sequence
Bam HI	1	A Bam 24	5′-AGCACTCTCCAGCCTCTCACCGAG-3′
		A Bam 12	5′-GATCCTCGGTGA-3′
	2	O Bam 24	5′-ACCGACGTCGACTATCCATGAACG-3′
		O Bam 12	5′-GATCCGTTCATG-3′
	3	E Bam 24	5′-AGGCAACTGTGCTATCCGAGGGAG-3′
		E Bam 12	5′-GATCCTCCCTCG-3′
Xba I	1	A Xba 24	5′-AGCACTCTCCAGCCTCTCACCGAT-3′
		A Xba 12	5′-CTAGATCGGTGA-3′
	2	O Xba 24	5′-ACCGACGTCGACTATCCATGAACT-3′
		O Xba 12	5′-CTAGAGTTCATG-3′
	3	E Xba 24	5′-AGGCAACTGTGCTATCCGAGGGAT-3′
		E Xba 12	5′-CTAGATCCCTCG-3′
Hind III	1	A Hin 24	5′-AGCACTCTCCAGCCTCTCACCGCA-3′
		A Hin 12	5′-AGCTTGCGGTGA-3′
	2	O Hin 24	5′-ACCGACGTCGACTATCCATGAACA-3′
		O Hin 12	5′-AGCTTGTTCATG-3′
	3	E Hin 24	5′-AGGCAGCTGTGGTATCGAGGGAGA-3′
		E Hin 12	5′-AGCTTCTCCCTC-3′

Note. Primer set 1 (A series) was used for preparing amplicon representations; set 2 and 3 (O series and E series, respectively) were used for odd and even hybridization-amplification.

Each tube (400 μl) contained, 67 m*M* Tris-HCl, pH 8.8 at 25°C, 4 m*M* $MgCl_2$, 16 m*M* $(NH_4)_2SO_4$, 10 m*M* β-mercaptoethanol, bovine serum albumin (100 μg/ml), 300 μ*M* each dATP, dGTP, dCTP and dTTP, 1 μ*M* 24-bp primer and 80 ng of DNA with ligated adaptors. After amplification, both driver and tester amplicons were digested with the same restriction endonuclease (10 units/μg) to cut the adaptors from the amplicons. For removal of the adaptor, 2.5 μg of tester amplicon DNA digests were subjected to electrophoresis through 2% agarose gel, and DNA fragments (150–1500 bp) recovered by using QIAEX II Gel Extraction Kits (QIAGEN Inc., Chatsworth, CA).

Three-Round Subtractive Hybridization and PCR Amplification (SHPCRA). In the first round of SHPCRA, each of the tester amplicons (500 ng) was ligated to new primers (Table I, primer set 2) following the same procedure for preparation of the amplicon and mixed with 40 μg of driver amplicon. The amplicon mixture was denatured at 95°C for 5 min in 4 μl of 3X EE buffer (30 m*M* EPPs and 3 m*M* EDTA). One μl of 5 *M* NaCl was added, and the amplicon mixture was hybridized at 67°C for 20 hr. After hybridization, 10% of the resulting DNA was amplified for 20 cycles under the same conditions. Amplified DNA was digested by the original restriction endonuclease and 100 ng of the digested DNA ligated to the third set of primers (Table I, primer set 3). Twenty-five ng of DNA ligated with the new set of adaptors were mixed with 40 μg of driver amplicon, and the hybridization-amplification procedure was repeated. A sample (50 ng) of the DNA digest obtained after the second round of SHPCRA was ligated to the second set of primers (Table I, primer set 2), and 100 pg of this material together with 40 μg of driver amplicon were taken for the third round of SHPCRA. Specifically amplified DNA *via* the three-round SHPCRA was used as a probe for Southern blot assay.

Results

No Specific Target DNA was Acquired After Three-Rounds of SHPCRA in Amplicons from DNAs of Mycoplasma-Transformed Cells. Amplicons of three different restriction enzyme digests for tester, driver, and model tester DNAs were prepared first (see Materials and Methods). The primer sets (set 1) that were used to prepare amplicons for each different restriction enzyme were shown in Table I. First round of SHPCRA tester amplicons of C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells were ligated to primer set 2 for each of the three different restriction enzymes (Table I) and mixed with 80-fold excessive amount of driver amplicon from control parental C3H cells. Unannealed tester amplicon DNAs were selectively amplified by PCR using primer set 2. Second round of SHPCRA PCR products of ester amplicons from C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells in the first round were digested with respective restriction enzymes, ligated to primer set 3 (Table I), and mixed again with 80-fold excessive amount of driver amplicon of control parental C3H cells. Unannealed tester amplicon DNAs were again amplified by PCR, this time using primer set 3. Third round SHPCRA PCR products of tester amplicons in the second round were digested with respective restriction enzymes, ligated to primer set 2, and mixed again with excessive amount of driver amplicon DNA. Unannealed tester amplicon DNAs were amplified the third time by PCR, using primer set 2. After three rounds of SHPCRA, no specific differential product was amplified in both the testers (C3H/Mf/P18-Cip and C3H/Mp/P18-Cip) of the *Bam* HI (Fig. 1,

Lanes b3 and c3), *Xba* I (Fig. 1, Lanes b3 and c3), or *Hind* III (Fig. 2, Lanes b3 and c3) amplicons.

Target DNAs were Amplified from DNAs of Model Testers Examined in Parallel as Positive Test Controls. A target DNA in the model tester of the *Xba* I amplicon was clearly amplified after the second round of SHPCRA (Fig. 1, Lane a2). This DNA (about 300 bp) was further amplified in the third round of SHPCRA (Fig. 1, Lane a3). Similarly, another target DNA was also identified after the second round of SHPCRA (Fig. 2, Lane a2) in the model tester of the *Hind* III amplicon. In the third round (Fig. 2, Lane a3) of SHPCRA, the target DNA (about 196 bp) was further enriched with reduction of background amplicons. Because of the limited cutting sites for endonuclease *Bam* HI in the highly A-T rich mycoplasmal DNA spiked in, no specific target DNA was identified after the second round of SHPCRA (Fig. 1, Lane a2) and the third round of SHPCRA (Fig. 1, Lane a3) in this model tester of the *Bam* HI amplicon.

Target DNAs Amplified from Model Testers were Confirmed to be the Spiked-In Mycoplasmal DNAs. After the third round of SHPCRA, target DNA fragments were excised from the model tester *Xba* I amplicon (Fig. 1, Lane a3) and *Hind* III amplicon (Fig. 2, Lane a3), ^{32}P-labeled, and used as probes in Southern blot analysis. The amplified target DNA from the *Xba* I amplicon was used to probe endonuclease *Xba* I digested genomic DNA isolated from *M. fermentans* (*incognitus* strain), *M. penetrans* (GTU-54 strain), C3H cells, C3H/Mf/P18-Cip, and C3H/Mp/P18-Cip cells (Fig. 3A). The amplified target DNA from the model tester *Hind* III amplicon was used to probe endonuclease *Hind* III-digested genomic DNA from *M. fermentans* (*incognitus* strain), *M. penetrans* (GTU-54 strain), C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells as well as cloned 2.2-Kb DNA (psb-2.2) of *M. incognitus* (15) (Fig. 3B). Southern blotting showed the probe from the *Xba* I amplicon hybridized with multiple bands of *M. fermentans* (*incognitus* strain) genomic DNA (Fig. 3A, Lane 2). There was no reaction to other DNAs from C3H cells or *M. penetrans.* It confirmed that the amplified *Xba* I target DNA was *M. fermentans* (*incognitus* strain) DNA that was originally added to C3H cells DNA serving as *Xba* model tester. The probe from the model tester of the *Hind* III amplicon was found to hybridize with genomic DNA of *M. fermentans* (*incognitus* strain) (Fig. 3B, Lane 2) and cloned *M. fermentans*-specific 2.2-Kb DNA (psb-2.2) (Fig. 3B, Lane 6), but not with other DNAs from *M. penetrans* or C3H cells. The *Hind* III target DNA amplified from the model tester hybridized intensely with the 196-bp *Hind* III fragment of psb-2.2. Figure 4 depicts the *Hind* III restriction enzyme map of the cloned *M. fermentans* 2.2-Kb DNA (15) that was added to C3H cells DNA serving as *Hind* III model tester. The 196-bp *Hind* III fragment of psb-2.2 was clearly the amplified target DNA in this RDA study.

Discussion

We reported that chronic persistent infections by *M. fermentans* and *M. penetrans* induced chromosome alterations and malignant transformation associated with overexpression of certain oncogenes in C3H cells. Differing from models of virus-mediated tumor formation, the mycoplasma-mediated oncogenesis required a long latency and demonstrated distinct stages of progression during transformation. In viral oncogenesis, the viral genome is found continuously present in the transformed cells (3–7). Its presence was required to maintain the characteristics of transformation in these cells. A possible viral role in the initiation of transformation but not maintenance was proposed (16). Because there was no "positive" evidence to support a "hit-and-run" mechanism in virus-mediated transformation, the hypothesis was not widely accepted.

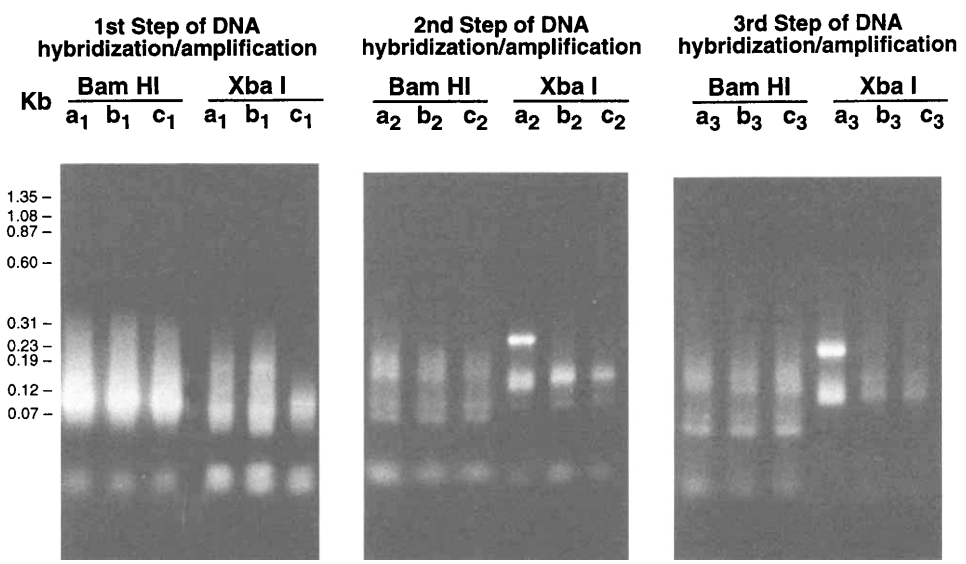

Figure 1. Agarose gel electrophoresis of amplicons from first, second, and third round of subtractive hybridization and PCR amplification (SHPCRA). Differential product from model tester of *Xba* I amplicon was identified in the 2nd round of SHPCRA (*Xba* I, Lane a2), which was further enriched in third round of SHPCRA (*Xba* I, Lane a3). In the study, there was no specific differential product detected in testers (*M. fermentans* and *M. penetrans* transformed C3H cells, C3H/Mf/P18-Cip, or C3H/Mp/P18-Cip cells) (*Xba* I, b2 or b3; c2 or c3). There was also no differential product identified in the model or other testers (C3H/Mf/P18-Cip or C3H/Mp/P18-Cip cells) of *Bam* HI amplicons after three rounds of SHPCRA (*Bam* HI, Lanes a3, b3 and c3). *Hae* III digested ϕX174 RF DNA was used as a molecular size marker.

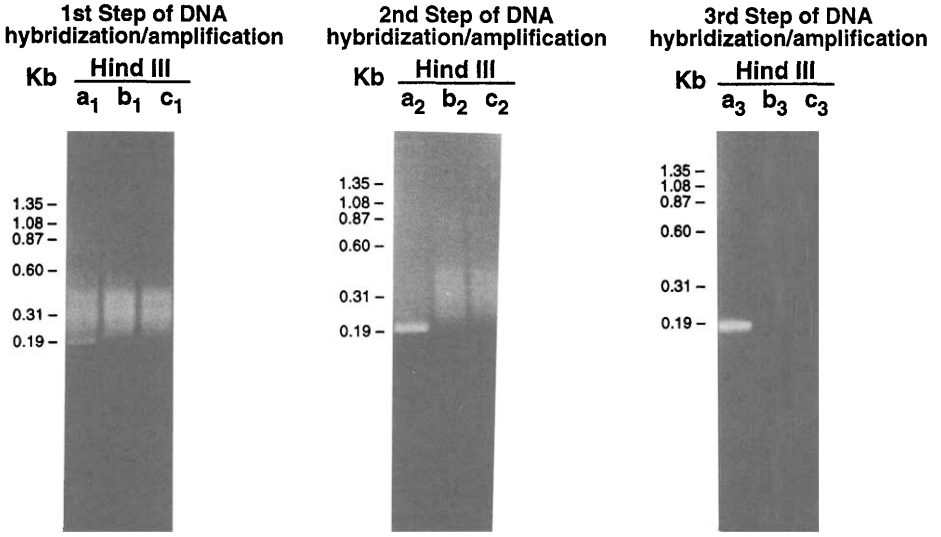

Figure 2. Agarose gel electrophoresis of differential products from *Hind* III amplicons after the first, second, and third round of SHPCRA. The differential product in the model tester of the *Hind* III amplicon was vaguely detected as a specific band in the first round of SHPCRA (Lane a1). This product or the target DNA band was further amplified in the second (Lane a2) and third found (Lane a3) of SHPCRA. In the study, there was no differential product detected in tester amplicons *M. fermentans* and *M. penetrans* transformed C3H cells, C3H/Mf/P18-Cip or C3H/Mp/P18-Cip, (Lanes b2 and b3; c2 and c3). *Hae* III digested ϕX174 RF DNA was used as a molecular size marker.

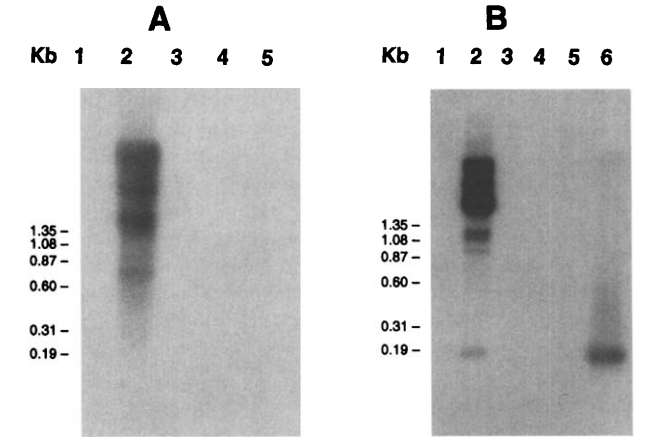

Figure 3. Agarose gel electrophoresis and Southern blotting of differential products form the *Xba* I amplicon. (A) Southern blotting of the agarose gel on a nylon membrane and probing by ^{32}P-labeled differential products from *Xba* I amplicon. The amplified target DNA was excised from model tester of *Xba* I amplicon (Fig. 1, *Xba* I, Lane a3) and used as probe. Multiple intensely positive bands were detected in genomic DNA of *M. fermentans* (Lane 2). No positive reaction to DNAs from C3H cells, C3H/Mf/P18-Cip, C3H/Mp/P18-Cip cells, or *M. penetrans* (Lanes 1, 3, 4 and 5, respectively) was found. *Hae* III digested ϕX174 RF DNA was used as a molecular size marker. (B) Southern blotting of the agarose gel on a nylon membrane and probing by ^{32}P-labeled differential products from *Hind* III amplicon. The amplified target DNA was excised from model tester of *Hind* III amplicon (Fig. 2, Lane a3) and used as probe. Multiple intensely positive bands were detected in genomic DNA of *M. fermentans* (Lane 2), and one 196-bp single band was detected in cloned 2.2-Kb DNA of *M. fermentans* (*incognitus* strain) (Lane 6). No positive reaction to DNAs from C3H cells, C3H/Mf/P18-Cip, C3H/Mp/P18-Cip cells, or *M. penetrans* (Lanes 1, 3, 4 and 5, respectively) was found. *Hae* III digested ϕX174 RF DNA was used as a molecular size marker.

Digest with HindIII:

Fragment Size	Left Overhang	Cut by Enzyme	From : To	Cut by Enzyme	Right Overhang
1897		5' end	1:1897	HindIII	[+ 4]
196	[+ 4]	HindIII	1964:2159	HindIII	[+ 4]
66	[+ 4]	HindIII	1898:1963	HindIII	[+ 4]
51	[+ 4]	HindIII	2160:2210	3' end	

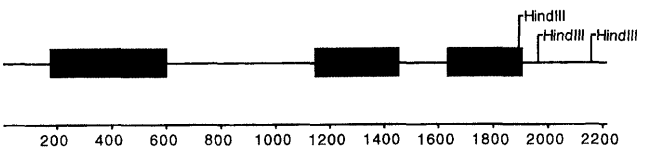

Figure 4. Schematic description of the *Hind* III restriction enzyme map derived from analysis of the nucleotide (nt) sequence of cloned 2.2-Kb DNA (Plasmid psb-2.2) of *M. fermentans* (*incognitus* strain) *Eco*RI 2.2-Kb DNA. The sequence of Plasmid psb-2.2 was previously reported (15). There are four cleaving areas ranging from (5′ end) 1 to 1897 base pair (bp), 1989 to 1963 bp, 1964 to 2159 bp, and 2160 to 2210 bp (3′ end) yielding estimated fragments of 1897 bp, 66 bp, 196 bp, and 51 bp, respectively.

On the other hand, oncogenic mechanism(s) produced by prolonged mycoplasmal infections was likely to be different from those by oncogenic viruses. In this study using an extremely sensitive technique coupled with appropriate controls, we showed that no stretches of DNA or fragments of gene(s) of mycoplasmal origin could be detected in the transformed mammalian cells induced by chronic infections of mycoplasmas. These permanently transformed cells apparently did not require presence of any mycoplasmal gene(s) to maintain transformed characteristics.

The limits of resolution in RDA are difficult to assess. This RDA technique essentially examined 150–1500-bp DNA digests from three different restriction enzymes. PCR would normally amplify DNA fragments of a few hundred bp much more effectively. How small of a foreign insert can be detected in DNA digests of a few hundred bp is very dependent on the differential ability of the subtractive hybridization. It should be reasonable to expect that an insert of more than 10 bp would significantly affect the efficiency or kinetics of hybridization for DNA fragments of this size. Naturally, examination of more different restriction enzyme digests would increase sensitivity of finding mycoplasmal (foreign) DNA in C3H cells.

What processes other than mycoplasmal DNA insertion could cause chromosomal changes and malignant transfor-

mation of mammalian cells? The actual mechanisms are still elusive, but clearly the process would require a prolonged course of infection by the mycoplasmas. It may be important to note that attachment or close interaction with mammalian cells by various bacterial agents could significantly alter a wide spectrum of signals transmitted from membranes to nuclei. It could also change cytoskeleton architecture in mammalian cells (17–19). Many of the transduced signals might trigger the pathways that promote continuous cell growth (20–22). In this context, mycoplasmas are found to be producing important cell membrane–acting enzymes that would surely affect membrane functions of mammalian cells (23–25).

Since wall-free mycoplasmas lack bacterial lipopolysaccharide endotoxins, infections by mycoplasmas do not normally produce acute pathogenicity seen in many bacterial infections. Although producing less acute illness in hosts, mycoplasmas often cause a chronic persistent infection. However, continuously transducing "altered" signals from the surface into nuclei of cells chronically infected by low virulent organisms may produce a unique form of pathogenesis including promotion of malignant transformation. Our experimental model showed prolonged infection with mycoplasmas in C3H cells induced overexpression of proto-oncogenes, which in turn, signal cells to replicate continuously and led to the morphological transformation (1, 2). Interestingly, for a long period of time (up to 11 weeks), these rapidly growing cells were still in a reversible stage of transformation. Removal of the stimulatory ligands, (i.e. mycoplasmas) by antibiotic eradication, rapidly reversed all the transforming properties of C3H cells, presumably by interdicting inappropriate signals transduced from the cell surface and restoring proper cytoskeleton arrangement.

In addition to receiving prolonged "altered" signals transduced from cell surface, our study showed chronic infections by mycoplasma also continued to introduce low grade cytotoxicity or damage to the mammalian cells, either directly at the cellular level or indirectly through production of harmful microbial metabolites. After a further prolonged period (18 weeks), chronically infected mammalian cells evidently had gradual accumulative damage or mutations to their chromosomes (1). Some cells with prominent chromosomal changes might have experienced a crucial mutation(s) that resulted in constitutive expression at high levels of both H-*ras* and c-*myc* proto-oncogenes (2). The chromosomal changes were apparently irreversible. These cells become permanently transformed. The cells with true growth advantage would quickly outgrow other cells that still depended on external stimulatory signals for growth and would soon be selected out in culture and be able to form tumors in animals.

In conclusion, our study suggests that chronic persistent infection by mycoplasmas of seemingly low pathogenicity or cytotoxicity may be important in promoting tumorous growth of mammalian cells. The mechanism of mycoplasma-mediated tumorigenesis is apparently different from that of oncogenic viruses. Presence of mycoplasmal DNA was not required to maintain the characteristics of transformation in these mammalian cells.

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