High-Level Expression of H-*ras* and c-*myc* Oncogenes in Mycoplasma-Mediated Malignant Cell Transformation (44104)

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Abstract. **C3H mouse embryo cells, which normally have low inherent spontaneous transformation, underwent malignant transformation while chronically infected with *Mycoplasma fermentans* or *Mycoplasma penetrans*. This mycoplasma-mediated oncogenic process had long latency (more than 7 weeks of persistent mycoplasmal infection) and showed multistage progression characterized by reversibility and irreversibility of malignant properties upon removal of *M. fermentans* from culture. Marked expression of H-*ras* and c-*myc* mRNA, but not N-*myc*, *src*, N-*ras*, or p53 mRNA, was found in the mycoplasma-transformed C3H cells that exhibited characteristic malignant properties of morphological changes and uncontrolled cell growth. However, at least up to the eleventh week of persistent mycoplasma infection, the marked expression of H-*ras* or c-*myc* mRNA in C3H cells depended on continued presence of the mycoplasma in culture. H-*ras* or c-*myc* mRNA rapidly declined to the undetectable low levels of nontransformed parental C3H cells, and all malignant properties of the once-fully-transformed C3H cells quickly reversed, if *M. fermentans* was eradicated from culture. In comparison, infection with *M. penetrans* for 7 or 11 weeks also induced a high level of H-*ras*, but not c-*myc*, mRNA expression in C3H cells. Despite having prominent amount of steady-state H-*ras* mRNA, these *M. penetrans*–infected C3H cells did not show any sign of malignant transformation. Thus, marked expression of H-*ras* gene alone was not sufficient to effect transformation in C3H cells. Interestingly, after a further prolonged (18 weeks) infection with either *M. fermentans* or *M. penetrans*, C3H cells revealed prominent chromosomal changes, expressed constitutively (with or without the presence of the transforming mycoplasmas) at high levels of both H-*ras* and c-*myc* mRNA and became permanently transformed. These cells were able to form tumors in animals.**

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In 1960s, it was reported that introduction of mycoplasmas into cultures of baby hamster kidney (BHK) cells produced immediate morphological transformation with a high soft agar cloning efficiency (1). Unfortunately, the BHK cells often had a high rate of spontaneous transformation. Decades later, in the 1980s, one other report was also made of rapid cell transformation, involving an arthropod spiroplasma (2). However, most scientists thought mycoplasms simply introduced confusing artifacts that mimic cell transformation (3). Although few believe that infections with mycoplasmas, like with some viruses, may transform mammalian cells into tumor cells, the possibility has great biological significance with apparent clinical implication and deserves more careful study. Moreover, there have been occasional clinical observations suggesting a possible association between infections by mycoplasmas and development of tumor-like lesions as well as true malignancies in humans (4–8).

Wall-free mycoplasmas are among the few prokaryotes that can grow in close interaction with mammalian cells, often silently, for a long period of time. It is also

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known that interactions on the surface of mammalian cells by a wide variety of ligands can trigger a cascade of mechanisms transducing cell growth-stimulating signals from membranes to nuclei (9, 10). We believe chronic and persistent infection with microorganisms with seemingly low virulence may gradually but significantly affect many important biological characteristics of mammalian cells. In a recent study, we experimentally introduced *Mycoplasma fermentans* (incognitus strain) and *Mycoplasma penetrans* into murine embryonic C3H10T1/2 (C3H) cells (11). C3H cells rarely undergo spontaneous transformation and are one of the few standard test systems for studying potential carcinogenic agents in animals or humans (12). *M. fermentans* and *M. penetrans* are the most common mycoplasmas associated with HIV infection or AIDS and are proposed to play a co-factor role in the disease process (13, 14).

As opposed to earlier reports (1, 2), we did not find infection by *M. fermentans* of *M. penetrans* induced rapid mammalian cell transformation. The C3H cells underwent transformation only after they had been persistently infected by the mycoplasmas for a prolonged period of time (latency). Furthermore, at least up to 11 weeks, malignant properties associated with cell transformation could be rapidly reversed upon antibiotic treatment to eradicate the mycoplasma in culture (11). This finding indicated that to maintain morphological transformation in mycoplasma-transformed C3H cells, for a rather long period of time, would require the continued presence of the "transforming mycoplasma" and that infection of the mycoplasma did not simply select out pre-existing subclones of malignant C3H cells in culture.

In this study, we attempted to identify possible molecular mechanisms responsible for producing the transforming properties in the mycoplasma-infected C3H cells. Because cell growth is normally regulated by a delicately balanced interplay among various oncogenes and antioncogenes, overexpression of certain oncogenes or loss of a tumor-suppressor gene may result in uncontrolled cell growth (9, 10, 15, 16). Total DNA was isolated from parental C3H cells and mycoplasma-transformed C3H cells that were able to grow into colonies on soft agar and formed tumors in animals, and analyzed by labeled probes of c-*myc*, N-*myc*, H-*ras*, N-*ras*, *src*, and p53 genes for changes such as gene amplification or rearrangement. Since no such a change was identified, we then compared the differences in expression of these oncogenes and tumor-suppressor gene in C3H cells at the distinct stages of malignant progression. We were particularly interested in the nature of differential expression of these oncogenes at the reversible and the irreversible stages of mycoplasma-mediated oncogenesis. Total RNA was extracted from C3H cells following varying periods (7, 11, or 18 weeks) of infection by *M. fermentans* or *M. penetrans* and was analyzed by labeled probes for c-*myc*, N-*myc*, H-*ras*, N-*ras*, *src*, and p53 mRNA, respectively. H-*ras* and c-*myc* oncogenes, that have long been implicated in human and animal carcinogenesis, were found to play important roles in cell transformation induced by chronic persistent infection by these mycoplasmas. There were apparently two different (induced or constitutive) forms of high-level H-*ras* and c-*myc* mRNA expression that were closely associated with the reversible or the irreversible states of mycoplasma-mediated transformation in C3H cells.

Material and Methods

Cells and Cell Culture. Malignant transformation of mouse embryonic C3H/10T1/2 cells during persistent infection with *M. fermentans* (Mf) and *M. penetrans* (Mp) was previously described in detail (11). Briefly, C3H cells infected with Mf or Mp after 7 weeks (1 week/passage), 11 weeks, and 18 weeks were termed C3H/Mf/P7, C3H/Mf/P11, and C3H/Mf/P18, or C3H/Mp/P7, C3H/Mp/P11, and C3H/Mp/P18, respectively. C3H/Mf/P7 cells exhibited phenotypic changes with malignant characteristics that became progressively more prominent with further prolonged infection (e.g., C3H/Mf/P11). In comparison, C3H/Mp/P7 and C3H/Mp/P11 did not reveal similar morphological transformation. However, all malignant changes found in C3H/Mf/P7 and C3H/Mf/P11 were reversible if mycoplasmas were eradicated by 3 weeks of treatment with the antibiotic ciprofloxacin (Cip). Thus, C3H/Mf/P7-Cip and C3H/Mf/P11-Cip reverted to a normal phenotype (11). Further persistent infection with either mycoplasma until 18 weeks resulted in irreversible transformation that no longer required the continued presence of mycoplasmal agents in cultures. C3H/Mf/P18-Cip and C3H/Mp/P18-Cip were permanently transformed cells which showed prominent karyotypic changes (11). In this study, all cells were cultured in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS, Gibco), with 5% CO_2 at 37°C. Quiescent cells were prepared by inoculating 9×10^5 cells in 25 ml of serum-containing media into 150-cm^2 culture flask and grown to confluence for 1 week in the original medium. Serum stimulation of quiescent cells was accomplished by changing the culture medium to fresh medium with 15% FBS, and the cultures were allowed to continue for different periods of time.

Preparation of DNA and RNA. DNA was isolated and purified from nearly confluent (Day 4) cultures of C3H/Mf/P18-Cip, C3H/Mp/P18-Cip, and C3H/P18-Cip cells by phenol extraction as previously described (17). RNA was isolated using RNAzol B (TEL-TEST, Inc., Friendswood, TX), according to the manufacturer's protocol. Cells (10×10^6) were first homogenized in 2 ml of RNAzol B. RNA was extracted from the cell homogenate (1 volume) by adding 0.1 volume of chloroform, and then precipitated by transferring the aqueous phase

to 1 volume of isopropanol. The RNA pellets were washed with 70% ethanol and dissolved in 500 μl TE buffer (10 m*M* Tris-HCl, pH 7.6; 1 m*M* EDTA). Prime RNase inhibitor (5 Prime → 3 Prime, Inc., Boulder, CO) was added to the prepared RNA samples (1 unit/30 μl) before they were stored in −70°C freezer. For the kinetic study of oncogene expression, RNA was isolated at each indicated time interval after fresh serum stimulation of the quiescent cells. To compare c-*myc* and H-*ras* oncogene expression in C3H cells undergoing reversible or irreversible stages of transformation (at 7, 11, or 18 weeks of persistent mycoplasmal infections), RNAs were isolated from quiescent cells 1 hr after serum stimulation for c-*myc* detection and 24 hr post serum stimulation for H-*ras* detection. In Southern blotting, DNA samples (10 μg/lane) were digested with either *Hin*dIII or *Eco*RI according to the manufacturer's recommendations (Gibco, BRL) and separated by electrophoresis on a 1% agarose gel. In Northern blotting, RNAs (25 μg/lane) were fractionated by electrophoresis in 1% formaldehyde agarose gel. Electrophoretically separated DNA or RNA was transferred onto nylon membranes by Southern blot then baked at 80°C for 30 min.

Probes, Labeling, and Hybridization. Oncogene DNA probes c-*myc* (1.4 kb, human c-*myc,* third exon), N-*myc* (1.0 kb, human N-*myc,* second exon), v-H-*ras* (0.73 kb, Harvey Rat Sarcoma Virus), N-*ras* (1.5 kb, human N-*ras,* 3′ end), v-*src* (0.8 kb, Avian Sarcoma Virus), and v-*foc* (1.0 kb, FBJ Osteosarcoma Virus Proviral DNA) as well as a positive control β-actin DNA probe (1.0 kb, human β-actin) were all obtained from Oncor (Gaithersburg, MD). The p53 probe (0.2 kb, human p53, sixth exon, PCR amplicon from human breast tissue) (18) was kindly provided by Dr. T. J. O'Leary's laboratory, Armed Forces Institute of Pathology (Washington, DC). The c-*jun* probe (1.0 kb) (19) was kindly provided by Dr. Shiaho Shih of University of Texas (Galvaston, TX). All probes were labeled with [α-^{32}P] dATP (Amersham Co., IL) to a specific activity of 1–3 × 10^9 cpm/μg using the multiprime DNA labeling system (20).

Filters with DNA or RNA samples were prehybridized in solutions containing 50% deionized formamide, 5× Denhart's solution, 0.1% SDS, 5× SSC, and 100 μg/ml freshly denatured salmon sperm DNA at 42°C for 1 hr. Hybridization was continued in the same solutions as prehybridization by adding freshly denatured DNA probes (1–3 × 10^6 cpm/ml) at 42°C for 24 hr. After hybridization, the filters were washed once in 2× SSC/0.1% SDS, twice in 0.1× SSC/0.1% SDS for 15 min per wash at ambient temperature, then in 0.1× SSC/0.1% SDS for 30 min at 56°C. Filters were autoradiographed using Kodak X-OMAT XAR-5 film at −70°C in the presence of intensifying screens, exposures varied from 3 days (for most Southern blots) to 7 days.

Measurement of Hybridized Signals. A Phosphor Imager (Molecular Dynamics, Sunnyvale, CA) was used to quantitate the oncogene messengers expressed in mycoplasma-transformed cells and control cells. Hybridized RNA filters were placed on the imaging plate and pressed tightly in a Dynamics Exposure Cassette. After exposure for the desired time at room temperature, the imaging plates were scanned in the Phosphor Imager and the intensity of the hybridization signal was quantitated using the Image Quant (21).

Results

Oncogene Amplification and Rearrangement. We examined c-*myc,* N-*myc,* H-*ras,* N-*ras,* and *src* oncogenes and the p53 tumor-suppressor gene for possible DNA changes in irreversibly transformed C3H cells after 18 weeks of infection with *M. fermentans* or *M. penetrans* followed by 3 weeks of ciprofloxacin treatment to eradicate the mycoplasms (C3H/Mf/P18-Cip or C3H/Mp/P18-Cip). The C3H cells that were cultured 18 weeks in parallel without mycoplasma infection and treated with ciprofloxacin for 3 weeks (C3H/P18-Cip) were used as the control. Both C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells were transformed and had prominent karyotypic changes (11). In Southern blot analysis, restriction-enzyme digests of DNAs from C3H/Mf/P18-Cip, C3H/Mp/P18-Cip, and C3H/P18-Cip cells with either *Hin*dIII or *Eco*RI exhibited identical bands for c-*myc,* N-*myc,* H-*ras,* N-*ras, src,* and p53 oncogenes. No detectable change in copy number was discerned between DNA from the transformed C3H cells and the control C3H cells, suggesting that amplification or DNA rearrangement of those oncogenes did not occur in these permanently transformed cells (Fig. 1).

Marked Increase of c-*myc* and H-*ras* mRNA Expression. Expression of the six oncogenes or the p53 anti-oncogene in these permanently transformed C3H cells induced by chronic infection with *M. fermentans* or *M. penetrans* was also examined. Total RNA was isolated from newly confluent cultures of C3H/Mf/P18-Cip, C3H/Mp/P18-Cip, and the control C3H/P18-Cip cells. Each of the six oncogenes or the anti-oncogene was [^{32}P]-labeled and used as a probe in Northern blot analysis. Production of c-*myc* and H-*ras* mRNA was found to be markedly enhanced in these cells permanently transformed by either *M. fermantans* or *M. penetrans.* In comparison, transcriptional products of N-*myc,* N-*ras, src,* and p53 genes were not found to be significantly increased in C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells (data not shown).

Kinetics of c-*myc* and H-*ras* Expression. To evaluate the kinetics and peaks of c-*myc* and H-*ras* mRNA expression in transformed C3H cells, we tested RNA isolated at different intervals after fresh serum stimulation of the quiescent C3H/Mf/P18-Cip, C3H/Mp/P18-Cip, and control C3H/P18-Cip cells (Fig. 2).

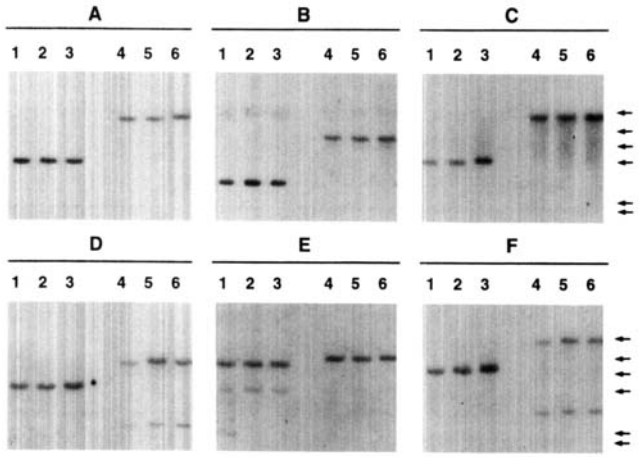

Figure 1. Southern blot analysis of oncogenes in permanently transformed C3H cells. DNAs (10 μg/lane), extracted from control C3H cells (C3H/P18-Cip) (Lanes 1 and 4) and transformed C3H cells infected with *M. fermentans* for 18 weeks and followed by ciprofloxacin treatment (C3H/Mf/P18-Cip) (Lanes 2 and 5) or with *M. penetrans* for 18 weeks followed by ciprofloxacin treatment (C3H/Mp/P18-Cip) (Lanes 3 and 6), were digested with restriction enzyme *Hin*dIII (Lanes 1–3) or *Eco*RI (Lanes 4–6) and fractionated on 1% agarose gels and transferred onto nylon membrane filters. The filters were individually hybridized with [^{32}P]-labeled oncogene probes: (A) c-*myc*, (B) N-*myc*, (C) H-*ras*, (D) N-*ras*, (E) *src*, and (F) p53. Arrows represent the position of molecular weight markers from λ DNA digested by *Hin*dIII (23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 kb).

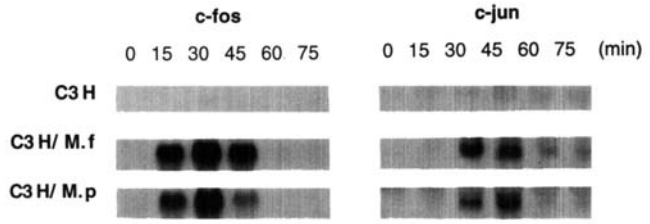

Figure 3. Expression of c-*fos* and c-*jun* in permanently transformed C3H cells. Total RNA (25 μg/lane), extracted from quiescent C3H/Mf (C3H/Mf/P18-Cip), C3H/Mp (C3H/Mp/P18-Cip), and control C3H (C3H/P18-Cip) cells at early short intervals following fresh serum stimulation were analyzed by Northern blot using radiolabeled c-*fos* and c-*jun* oncogene probes. Peak response of c-*fos* and c-*jun* appeared at 30 and 45 min, respectively, following serum stimulation.

The peak steady-state mRNA expression for c-*myc* and H-*ras* appeared at 1 hr and 24 hr after fresh medium/serum stimulation, respectively, in both C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells. The duration of increased H-*ras* mRNA expression lasted for a few hours, much longer than the increased c-*myc* mRNA expression, which lasted approximately 30 min. Production of c-*myc* mRNA in these cells remained low for at least another 30 hr after the initial peak. In comparison, there was no detectable expression of mRNA for either of the two proto-oncogenes in control C3H/P18-Cip cells following fresh serum stimulation (Fig. 2).

Induction of c-*myc* mRNA and Other Nuclear Oncogenes Expression. Induction of c-*myc* mRNA expression is often preceded by induction of two other proto-oncogenes, c-*fos* and c-*jun,* that function as transcriptional factors (22, 23). Thus, we also examined the c-*fos* and c-*jun* mRNA expression in the mycoplasma-transformed C3H cells. Both quiescent C3H/Mf/P18-Cip and C3H/Mp/P18-Cip cells, but not control C3H/P18-Cip cells, showed an extremely early sharp induction of c-*fos* and c-*jun* mRNA following fresh serum stimulation. Induction of c-*fos* mRNA expression started at 15 min and peaked at 30 min. Induction of c-*jun* mRNA quickly followed, starting at 30 min and reaching peak level at 45 min (Fig. 3).

Induction of c-*myc* and H-*ras* mRNA Expression in C3H Cells at Reversible and Irreversible Stages of Mycoplasma-Mediated Transformation. To compare c-*myc* and H-*ras* mRNA produced in C3H cells during reversible or irreversible stages of transformation, we examined RNA isolated from C3H cells after 7, 11, or 18 weeks of persistent infections by *M. fermentans* or *M. penetrans.* Morphologically transformed C3H cells after 7 or 11 weeks of *M. fermentans* infection (C3H/Mf/P7 or C3H/Mf/P11) were producing large amounts of H-*ras* and c-*myc* mRNA (Fig. 4). In comparison, C3H cells after 7 or 11 weeks of *M. penetrans* infection (C3H/Mp/P7 or C3H/Mp/P11), exhibiting no abnormal morphological changes, were also producing large amounts of H-*ras* mRNA, but no detectable c-*myc* mRNA (Fig.

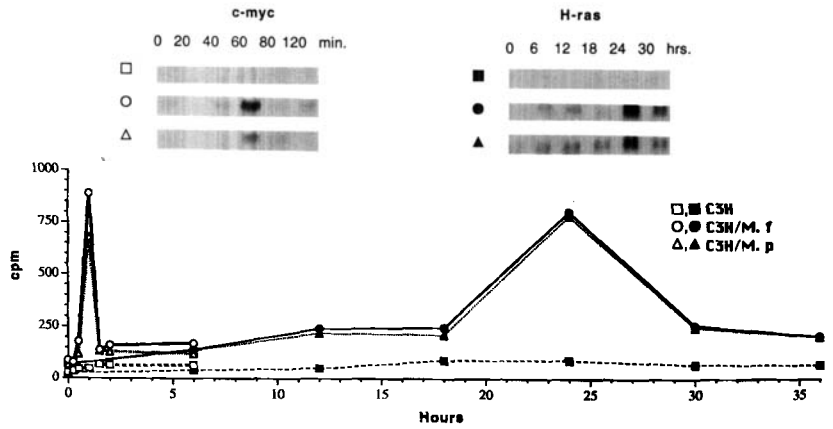

Figure 2. H-*ras* and c-*myc* expression in C3H cells permanently transformed by prolonged *M. fermentans* or *M. penetrans* infection. Total RNA (25 μg/lane), isolated from quiescent C3H/Mf (C3H/Mf/P18-Cip), C3H/Mp (C3H/Mp/P18-Cip), and the control C3H (C3H/P18-Cip) cells at different intervals following fresh serum stimulation were analyzed by Northern blot using [^{32}P]-labeled c-*myc* and v-H-*ras*. Peak response of c-*myc* and H-*ras* appeared at 1 and 24 hr, respectively, following serum stimulation.

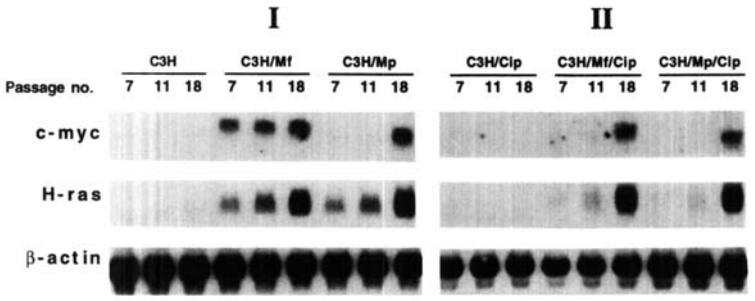

Figure 4. Northern blotting of c-*myc* and H-*ras* expression in C3H cells at different stages of transformation after different duration of mycoplasmal infections. Total RNA (25 μg/lane) isolated from C3H, C3H/Mf, and C3H/Mp cells at 7, 11, and 18 weeks, with continuous presence of mycoplasmas in the infected cultures (I) and after mycoplasmal eradication from the infected cultures with Cip treatment (II), were hybridized with [^{32}P]-labeled c-*myc* or H-*ras* probes. After hybridization, the filters were stripped in boiling wash solution and rehybridized with [^{32}P]-labeled β-actin.

4). Quantifying mRNA of these oncogenes showed a progressive increase in induction of both H-*ras* and c-*myc* mRNA expression in C3H/Mf cells and H-*ras* m-RNA expression in C3H/Mp cells over time for either mycoplasmal infection (Table I).

At least up to the eleventh week of mycoplasmal infection, the marked increase of c-*myc* and H-*ras* mRNA expression in C3H/Mf cells and H-*ras* mRNA expression in C3H/Mp cells clearly depended upon the continued presence of the mycoplasmas in culture. H-*ras* and/or c-*myc* mRNA in C3H/Mf/P11 cells and C3H/Mp/P11 cells quickly fell back to normal undetectable levels if the mycoplasmas were eradicated from the cultures by ciprofloxacin (Fig. 4 and Table I). Rapid decline of H-*ras* and c-*myc* mRNA closely coincided with rapid reversion of malignant characteristics in C3H/Mf/P7-Cip and C3H/Mf/P11-Cip cells (11). In contrast, the permanently transformed C3H cells that were known to have many chromosomal change (11), induced by more than 18 weeks of persistent infections by either *M. fermentans* or *M. penetrans* (C3H/Mf/P18 and C3H/Mf/P18-Cip; C3H/Mp/P18 and C3H/Mp/P18-Cip), continued to produce large quantities of H-*ras* and c-*myc* mRNA with or without the presence of the mycoplasmas in culture. To assure that the study was based on a similar amount of cellular mRNA tested, the RNA filter hybridized with either c-*myc* or H-*ras* oncogene probes was stripped in boiling wash solution and re-hybridized by [^{32}P]-labeled β-actin (Fig. 4).

Discussion

Activation of various oncogenes or inactivation of tumor-suppressor genes by mutation, amplification, translocation, or deletion of these genes is often associated with altered patterns of DNA fragments produced by digestion with restriction enzymes (15, 24–26). We first examined possible changes of c-*myc*, N-*myc*, H-*ras*, N-*ras*, *src*, and p53 genes in DNA of C3H cells permanently transformed by persistent infection (more than 18 weeks) with *M. fermentans* or *M. penetrans*. Despite obvious karyotypic alterations in these irreversibly transformed cells, we did not find any amplification or rearrangement of these oncogenes in Southern blot analysis of the *Hin*dIII- and *Eco*RI-digested DNA frag-

Table I. Relative Increase of c-*myc* and H-*ras* Oncogenes Expression in C3H Cells after Varying Periods of Mycoplasmal Infections

	Increase (× folds) at different passage number with mycoplasmas		
	7	11	18
I. Persistent infection with mycoplasmas:			
c-*myc*:			
C3H/*M. fermentans*	5.3 ± 0.7	6.2 ± 0.4	7.9 ± 1.1
C3H/*M. penetrans*	1.1 ± 0.1	1.2 ± 0.3	7.3 ± 1.1
H-*ras*:			
C3H/*M. fermentans*	7.3 ± 0.1	10.0 ± 0.3	16.1 ± 0.8
C3H/*M. penetrans*	7.1 ± 0.1	10.3 ± 0.3	15.7 ± 0.4
II. After eradication of mycoplasmas:			
c-*myc*:			
C3H/*M. fermentans*/Cip	1.1 ± 0.3	1.3 ± 0.1	9.2 ± 1.4
C3H/*M. penetrans*/Cip	0.9 ± 0.0	1.3 ± 0.1	6.8 ± 0.6
H-*ras*:			
C3H/*M. fermentans*/Cip	3.8 ± 0.4	5.5 ± 0.6	19.2 ± 0.1
C3H/*M. penetrans*/Cip	3.1 ± 0.7	5.0 ± 0.9	16.7 ± 0.7

Note. Relative increase of oncogene messagers (× folds) is calculated as the quantitative ratio between the amount of each oncogene messenger identified in C3H cells at the 7th, 11th, or 18th passage for each mycoplasma and the amount identified in mycoplasma-free C3H cells with the same number of parallel passages in culture. The results are presented as mean ± SD from two independent experiments.

ments. However, marked induction of mRNA expression for c-*myc* and H-*ras* proto-oncogenes was found to be associated with transformation of C3H cells induced by chronic mycoplasmal infections. Our study showed that, following serum stimulation, there was a temporal induction of early growth-related proto-oncogenes, c-*fos* and c-*jun*, preceding induction of the c-*myc* mRNA expression in the mycoplasma-transformed cells. Induction of c-*myc* mRNA was in turn followed by marked expression of H-*ras* mRNA and the onset of DNA replication. In comparison, expression of both c-*myc* and H-*ras* proto-oncogenes was almost undetectable in similarly serum-stimulated quiescent nontransformed C3H cells that had been passed in parallel without infection of mycoplasma.

As opposed to cell transformation induced by oncogenic viruses, mycoplasmas evidently did not induce malignant cell transformation by integrating their DNA into the mammalian host genome (27) (details will be published separately), required a long incubation time, and had progressive different stages. What clearly set the distinct stages of mycoplasma-mediated transformation apart were (i) differences in the kinetics of their induction effects (early versus late), (ii) alteration of cell chromosomes (with or without), and (iii) the ultimate nature of transformation (reversible versus irreversible). Although the molecular mechanism(s) of transformation remained unclear, in this study we revealed that cell transformation was effected through activation of H-*ras* and c-*myc* oncogenes. Like the permanently transformed C3H cells, morphologically transformed C3H cells during the reversible stage also exhibited a marked induction of H-*ras* and c-*myc* mRNA expression. However, contrary to the expression at constitutive high levels in the irreversibly transformed C3H cells, high-level expression of H-*ras* and c-*myc* genes depended on the continued presence of *M. fermentans* in culture. Both c-*myc* and H-*ras* mRNA quickly dissipated upon eradication of the mycoplasma from the cultures, closely coinciding with the rapid reversion of morphologic transformation of C3H cells. Thus, there were apparently two different forms (or two different mechanisms) in induction of H-*ras* and c-*myc* mRNA expression in C3H cells following the long course of persistent *M. fermentans* infection.

The reversible (or induced) and the irreversible (or constitutive) forms of c-*myc* and H-*ras* mRNA overexpression appeared to be, at least in part, responsible for the earlier, still reversible and the later, irreversible malignant transformation of C3H cells. Moreover, continued expression of both H-*ras* and c-*myc* proto-oncogenes at high levels is evidently necessary in maintaining malignant characteristics of C3H cells. *M. penetrans*–infected C3H cells, (examined at 7 and 11 weeks), while producing a large quantity of H-*ras* mRNA (but little c-*myc* mRNA), did not induce malignant transformation. Thus, H-*ras* mRNA overexpression alone was not sufficient to effect morphologic transformation in C3H cells. In this context, synergistic cooperation between c-*myc* and H-*ras* oncogenes in animal as well as human carcinogenesis has long been noted (28–30).

A schematic representation summarizes the mycoplasma-mediated cell transformation (Fig. 5). Persistent infection with *M. fermentans* for more than 6 weeks may have gradually perturbed the proliferation-signaling pathway and induced excessive production of proto-oncogenes c-*myc* and H-*ras* mRNA resulting in morphological transformation and extensive cell growth. Maintaining these malignant properties, however, requires the continued presence of the transforming mycoplasma. Once *M. fermentans* is removed from culture, both H-*ras* and c-*myc* mRNA rapidly decline, and transformed characteristics of C3H cells are soon reversed. A separate effect of the mycoplasma, requiring more time, causes genomic instability detectable as prominent chromosomal changes in the C3H cells. The mycoplasmal effects on mammalian cell genetic stability and chromosomal changes have previously been observed both in primary cultures of normal human cells and in immortalized human cell lines (31, 32). This second event may have gradually accumulated mutations in critical genes such as those related to the replication-signaling pathway and also in genes involved in DNA repair or in maintaining DNA fidelity. These genetic changes are clearly irreversible. Cells that constitutively express genes signaling replication such as H-*ras* and c-*myc* proto-oncogenes due to mutation(s) would now have a greater growth advantage and be preferentially selected. The clonal selection can clearly be seen in our study, with 40% or 80% of the transformed cells sharing the same translocation marker chromosome (11). These transformed cells, having acquired an hereditary tendency toward genetic instability and biological diversity, are also more likely to grow *in vivo* and form tumors in animals. This *in vitro* oncogenesis model mediated by chronic persistent infection of mycoplasmas has a long latency and a multistage malignant progression, the paradigm in naturally occurring human cancers.

It is important to point out that the true clinical significance for this *in vitro* experimental model is still not clear. Our study demonstrated the biological significance of the chronic, persistent nature of infections by low virulent microbes. However, the "threshold" of microbial load needed for the process is not known. In our infected cultures, the mycoplasma titers fluctuated from 10^4 to 10^6 CCU/ml (inoculation titer was 10^3 CCU/culture) (11). Will the 10^3 to 10^4 CCU/ml microbes commonly found in patients' sputum or urine be sufficient to trigger the process? On the other hand, it is difficult to determine the actual load or titers of mycoplasmas that exhibit strong cell-attachment properties and that colonize on the epithelium surface of bronchioles or of urogenital tracts rather than growing freely in alveolar

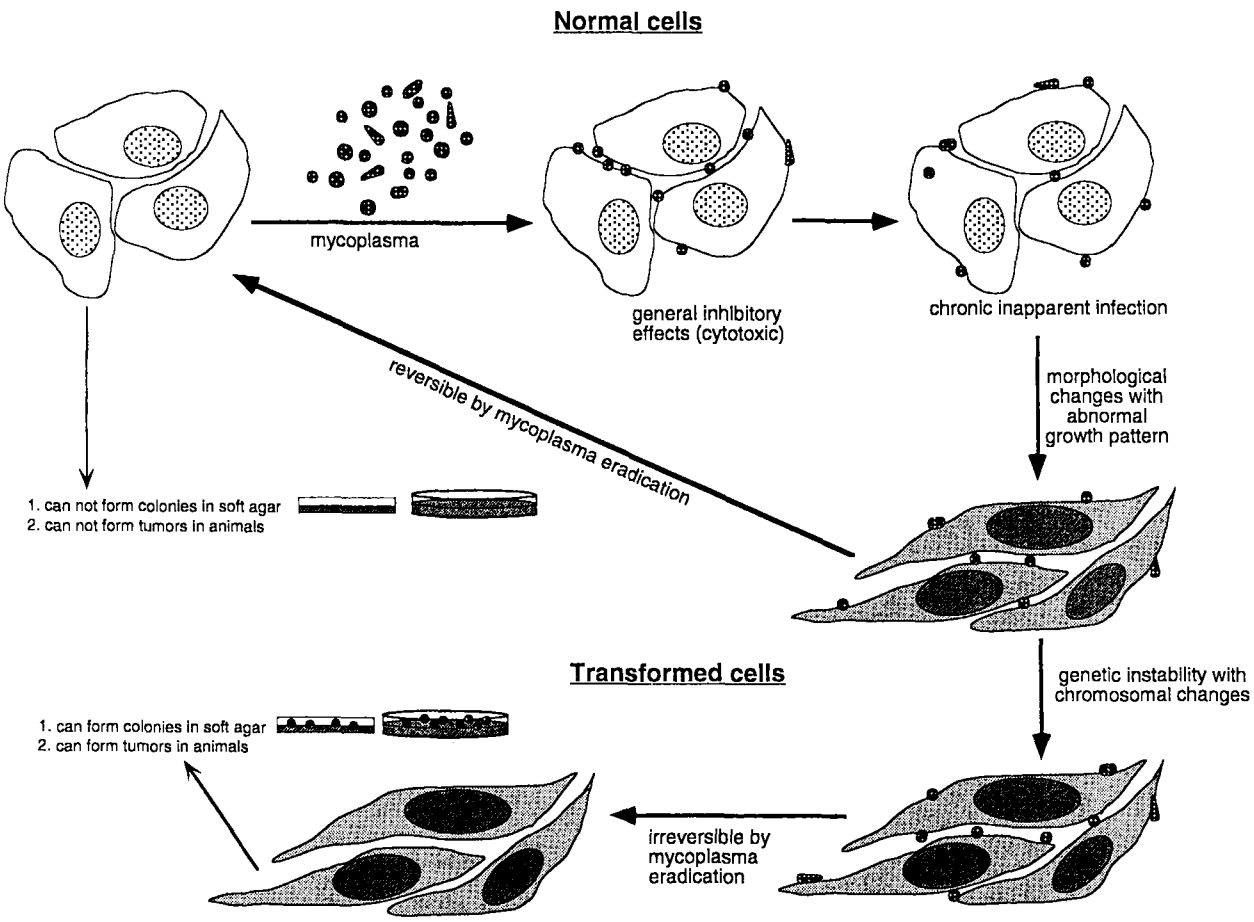

Figure 5. Schematic description of the multistage progression of mycoplasma-mediated transformation in C3H cells.

exudate or in urines. Furthermore, the *in vivo* conditions are much more complex. Many other active players are present. Mycoplasms are potent inducers for various inflammatory cytokines, NO, and/or superoxides (33, 34). And inflammatory cytokines may play an important role in certain neoplastic process, as proposed by some studies (35, 36). Also, in this context, other "flora" and different "substrates" that are simply not present in our pure culture system or in our media exist in *in vivo* conditions. The metabolites that could be produced by the mycoplasmas are expected to be quite different. It is impossible to assess the effects of these metabolites at the present. The ultimate clinical significance awaits well-designed epidemiological studies and subsequent development of an animal model.

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