acinar and ductal cells may derive from a common precursor, these transgenic mice should serve as a useful model even though the majority of human pancreatic tumors are diagnosed to be of ductal origin (*1*). In addition, the progression of these murine pancreatic tumors resembles several human hereditary malignancies, for example, familial adenomatous polyposis coli (*15*), and the multiple endocrine neoplasia syndromes (*16*). In each of these genetic diseases as well as other malignancies, such as Barrett's syndrome in the esophagus (*17*), adenomas characterized by hyperplasia and normal cellular DNA content become more dysplastic and eventually develop into carcinomas; malignant transformation is associated with the appearance of tetraploid and aneuploid cells. Thus, these ELSV transgenic lines may also provide a useful model for studying progression of other human neoplasms with similar properties.

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Clonal Analysis of Human Colorectal Tumors

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The clonal composition of human colorectal tumors was studied by means of restriction fragment length polymorphisms (RFLPs). First, X-linked RFLPs were used to examine the pattern of X chromosome inactivation in colorectal tumors of females. All 50 tumors examined showed monoclonal patterns of X chromosome inactivation; these tumors included 20 carcinomas as well as 30 adenomas of either familial or spontaneous type. Second, RFLPs of autosomes were used as clonal markers to detect the somatic loss or gain of specific chromosomal sequences in colorectal tumors. Among other changes, it was found that somatic loss of chromosome 17p sequences occurred in over 75 percent of the carcinomas examined, but such loss was rare in adenomas. These data support a monoclonal origin for colorectal neoplasms, and suggest that a gene on the short arm of chromosome 17 may be associated with progression from the benign to the malignant state.

ALTHOUGH THE ETIOLOGY AND mechanisms of development of human cancer are largely unknown, all hypotheses must incorporate observations concerning the clonality of neoplastic cell populations. Mutational theories of carcinogenesis predict that neoplasms will have a monoclonal composition (*1*). Alternative mechanisms for tumor formation, such as those involving aberrant differentiation processes or field effects, could lead to neoplasms with a polyclonal composition (*2*). At present, knowledge of the clonality of human tumors is incomplete. Many tumors, particularly those of hemopoietic or lymphopoietic origin, have been shown to be monoclonal (*3*), while other tumors appear to be polyclonal (*4–6*).

Colonic neoplasms provide a nearly ideal system to investigate the clonal composition of human cancer. Neoplasms of all stages can be readily identified histologically, from very small benign adenomas to large invasive carcinomas. Furthermore, colonic tumors occur in both heritable and spontaneous forms. Previous studies with isoenzymes of glucose-6-phosphate dehydrogenase (G6PD) have shown that one colorectal carcinoma (*5*) and seven colonic adenomas (*6*) were polyclonal. However, the possible presence of nonneoplastic stromal and epithelial elements within tumors complicates the interpretation of these studies.

We have examined the clonal composition of human colorectal tumors with the aid of two technological advances. First, we used DNA polymorphisms rather than protein polymorphisms. Our rationale was that each normal and neoplastic cell contributes approximately the same amount of DNA; this situation is unlike that potentially obtained by isozyme analysis (for example, G6PD) where the contribution from neoplastic and nonneoplastic cells may be disproportionate. Second, DNA was prepared from cryostat sections of tumors (*7, 8*). Histopathological analysis of alternate cryostat sections provided a reliable estimate of the relative contributions of neoplastic and nonneoplastic cells to the tissue component (that is, DNA) analyzed.

Restriction fragment length polymorphisms (RFLPs) were used to study X chromosome inactivation in colorectal carcinomas from 20 females; representative results are shown in Fig. 1. Analysis of DNA from normal colonic mucosa of a female heterozygous for an RFLP within the phosphoglycerate kinase (PGK) gene (*9, 10*) demonstrated two polymorphic alleles, designated 1 and 2, after Bst XI and Pst I digestion

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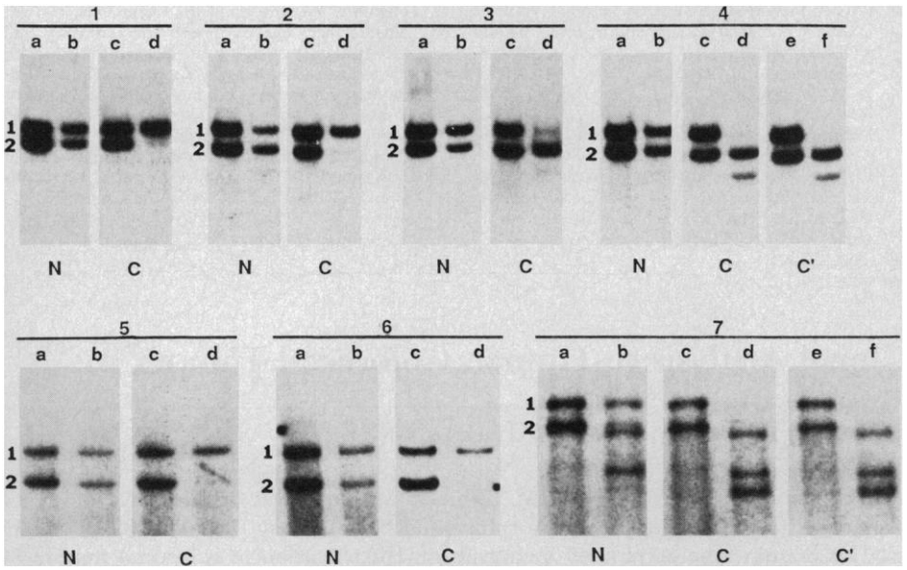

Fig. 1. Clonal composition of colorectal carcinomas determined by X chromosome inactivation analysis with cloned, polymorphic X chromosomal genes. RFLPs were used to distinguish the maternal from the paternal alleles of X chromosome genes, and methylation differences, as shown by Hpa II digestion, between active and inactive X chromosome genes were used to distinguish which of the chromosomes was inactivated. DNA was isolated from normal colonic mucosa (N) or from cryostat sections of carcinoma (C) of seven patients. Protocols used for clonal analysis with X-linked genes are as described (*9, 10*). Briefly, high molecular weight DNA was prepared from normal colonic mucosa or from cryostat sections of tumor specimens containing regions of carcinoma (*7, 8*). For analysis with the PGK probe, 8 to 10 μg of DNA was digested with Bst XI and Pst I (panels 1) or Bgl I, Bgl II, and Eco RI (panels 2 to 6). Subsequently, the DNA was divided into two equal aliquots; one aliquot was not digested further (panels 1 to 6, lanes a, c, and e), and the other aliquot was digested with Hpa II (panels 1 to 6, lanes b, d, and f). For analysis with the HPRT probe, the DNA was digested with Bam HI and Pvu II and either not digested further (lanes 7a, 7c, and 7e) or digested with Hpa II (lanes 7b, 7d, and 7f). Electrophoresis, Southern transfer, and hybridization to PGK or HPRT gene sequences were performed as described (*9*). The 1.7-kb and 1.3-kb Bgl I alleles (or 1.05- and 0.9-kb Bst XI alleles) of the PGK gene, and similarly the 18-kb and 12-kb Bam HI alleles of the HPRT gene, are designated to the left of each panel as "1" for the larger allele and "2" for the smaller. The fragments produced by Hpa II digestion are smaller in size than alleles 1 and 2; some of them are visible in lanes b, d, and f. The ratio of intensities between allele 1 and allele 2 after Hpa II digestion indicates the clonal composition of the cell population studied. C and C′ in panel 4 (and similarly in panel 7) represent DNA preparations, obtained by the cryostat sectioning technique, from two different regions of a single carcinoma specimen.

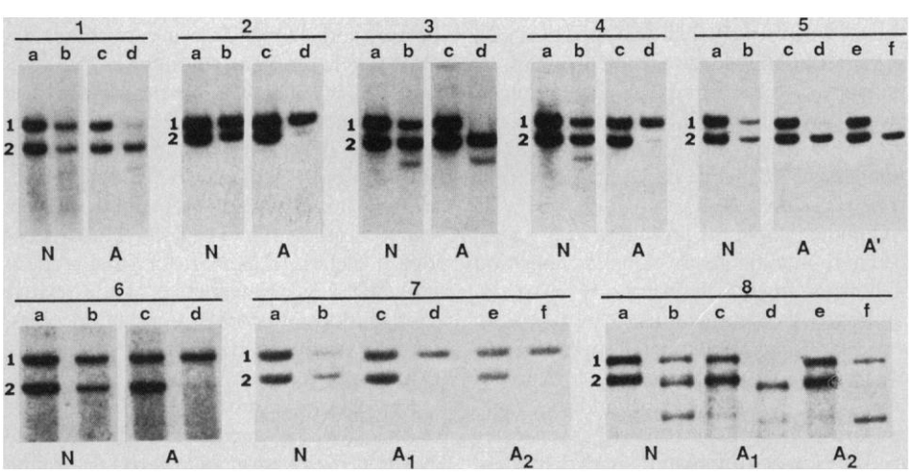

Fig. 2. Clonal composition of colorectal adenomas. Representative autoradiographs of Southern hybridization experiments using the PGK gene probe (panels 1 to 7) or the HPRT gene probe (panel 8) are shown. DNA was isolated from normal colonic mucosa (N) or cryostat sections of adenoma (A) of eight patients. In panel 5, A and A′ designate DNA preparations from two different regions of the same adenoma. In panels 7 and 8, A_1 and A_2 designate DNA prepared from cryostat sections of different adenoma specimens. The polymorphic alleles for each probe are designated "1" and "2" as described in the legend to Fig. 1. Preparation of DNA, restriction endonuclease digestion (lanes a to f) and Southern hybridizations to radiolabeled probes were as described in the legend to Fig. 1.

(Fig. 1, lane 1a and legend to Fig. 1). Subsequent digestion of the DNA with Hpa II, a methylation-sensitive restriction endonuclease, revealed the pattern of X chromosome inactivation (see legend to Fig. 1). In this normal tissue, a polyclonal pattern of X chromosome inactivation was observed: allele 1 was active in approximately half of the cells and allele 2 was active in the other half, as reflected by the fact that the ratio of intensities between allele 1 and allele 2 was approximately 1:1 after Hpa II digestion (Fig. 1, lane 1b). In contrast, analysis of DNA from cryostat sections of this patient's carcinoma revealed a monoclonal pattern: Hpa II digestion resulted in a nearly complete loss of allele 2, with a ratio of intensities between allele 1 and allele 2 in excess of 9:1 (lane 1d). Similar results were seen in studies of 19 carcinomas of other patients when the analysis was performed either with the PGK gene probe (examples in Fig. 1, panels 2 to 6) or with a probe for another X chromosome gene, the hypoxanthine phosphoribosyltransferase (HPRT) gene (examples in Fig. 1, lanes 7a to 7f). In three cases, we observed partial or total loss of one X chromosome in the carcinoma sample; in these cases, the remaining X chromosome was active, as expected (*11*). In 17 of the 20 carcinomas, DNA was prepared from cryostat sections of at least two widely separated areas of tumor. The different DNA preparations from a single carcinoma specimen always exhibited inactivation of the same X chromosome (Fig. 1, lanes 4c to 4f and lanes 7c to 7f).

Monoclonal patterns of X chromosome inactivation were seen in DNA preparations from cryostat sections of all 30 adenomas studied (examples in Fig. 2); 12 adenomas were of spontaneous type, and the other 18 were obtained from three patients with familial adenomatous polyposis syndrome. In all cases tested, DNA preparations from different areas of the same adenoma specimen showed inactivation of the same X chromosome (for example, lanes 5c to 5f in Fig. 2). While the different adenoma specimens of patients with familial adenomatous polyposis syndrome each exhibited a monoclonal pattern (Fig. 2, panels 7 and 8), the X chromosome that had been inactivated varied between different adenoma specimens of individual patients (Fig. 2, lanes 8d and 8f).

The basis for a further approach to study the clonal composition of these tumors is that karyotypic analyses of colorectal cancers have sometimes shown nonrandom gains and losses of chromosomes (*12*). In particular, some cytogenetic studies of colorectal cancers have shown frequent deletions of the short arm of chromosome 17 (*13*). The loss of normal cellular sequences in retinoblasto-

mas (*14*) and in other tumors (*8, 15*) is a clonal event that is believed to contribute to development of these neoplasms. Therefore, we sought to determine if we could demonstrate similar clonal losses of chromosome sequences in colorectal tumors.

Thirty-four carcinoma samples were examined for somatic loss of chromosome 17p sequences by means of RFLPs specific for this chromosome. Figure 3 shows representative Southern blots of DNA from normal and carcinoma tissue hybridized to pYNZ22, a probe that detects a highly polymorphic locus on chromosome 17p (*16*). DNA from carcinomas of 76% of patients demonstrated loss of one allele (Fig. 3, lanes 2C to 12C, and Table 1). When present, the chromosomal loss appeared to be clonal; that is, present in at least 90% of the neoplastic cells within the carcinoma specimen, as assessed by comparison of densitometric tracings of Southern blots with histopathological analysis of cryostat sections. In seven carcinomas, the loss of one allele appeared to be associated with the duplication of the retained allele, resulting in homozygosity for chromosome 17p sequences (Table 1 and examples in Fig. 3, panels 9 to 11).

We also examined RFLP patterns with probes specific for loci on chromosomes 1, 7, 11, 13, 16, and 20. Although some clonal changes were noted with these probes, loss of heterozygosity was observed in only 0 to 12% of the informative cases (Table 1). In addition, other chromosomal markers were found to be present in three copies in some carcinomas, but no carcinoma was found to have more than two copies of the 17p markers studied. The specificity of the loss of heterozygosity for chromosome 17p sequences was further emphasized by the finding that sequences on 17q were lost much less frequently (Table 1).

It was of interest to determine the relative time of occurrence of the loss of chromosome 17p sequences with respect to different stages of colorectal tumorigenesis. For this purpose we examined the DNA obtained from 30 adenomas from patients who were heterozygous for 17p markers (*17*). Examination of the DNA from these adenomas revealed that only one of the 30 adenomas had lost a chromosome 17p allele in a clonal fashion (*18*).

Although many carcinomas arise from preexisting adenomas (*19*), the adenomatous elements often have been replaced by carcinoma by the time the cancer has been diagnosed. However, in three cancers from this study which had lost chromosome 17p sequences, residual adenoma could still be identified within the tumor specimen. DNA was isolated by the cryostat sectioning technique from a region of carcinoma and a region of adenoma in each of these three cases. In two of the cases, the adenomatous cells within the tumor specimen had not lost a 17p allele (Fig. 3, lane 12A), suggesting that the loss of chromosome 17p sequences was associated with the change from adenoma to carcinoma. In the third case, the adenoma had lost the same allele as was lost in the adjoining carcinoma (*11*). This case showed more severe dysplasia in the adenoma than the other two cases.

Our results using X-linked and autosomal RFLPs are consistent with cytogenetic studies demonstrating clonal chromosomal abnormalities in many colorectal carcinomas (*12, 13*) and in some adenomas (*20*). Our data contrast, however, with the previously noted studies in which G6PD isozymes were used that suggested a polyclonal composition for both inherited adenomas (*6*) and one carcinoma (*5*). The results of Hsu *et al.* (*6*) demonstrating polyclonal composition of adenomas from patients with familial adenomatous polyposis syndrome may reflect contaminating nonneoplastic elements present in an otherwise monoclonal neoplastic lesion; the nonneoplastic elements must be removed for accurate analysis, as noted above. The finding of a patient with a polyclonal primary carcinoma and individual metastatic lesions containing either the paternal or maternal X chromosome activated (*5*) is more difficult to reconcile with our results. Among other interpretations, the unusually young age of the patient (32 years old) suggests that this case may not be representative of colorectal neoplasia in general.

The presence of a monoclonal pattern of X chromosome inactivation does not prove that the tumor actually originated from one

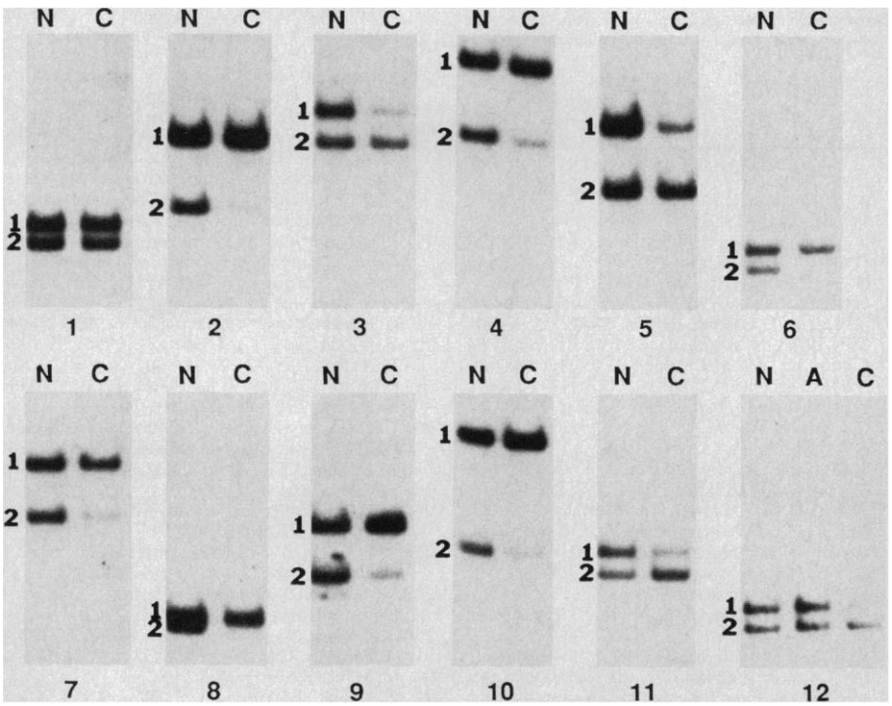

Fig. 3. Clonal loss of chromosome 17p sequences in colorectal carcinomas. Shown in the figure are autoradiographs of Southern hybridization experiments wherein a 17p gene probe, pYNZ22 (*16*), was hybridized to Bam HI-digested DNA from normal colonic mucosa (N) or DNA from cryostat sections of colorectal carcinoma (C) or adenoma (A) from 12 patients. In panel 12, the DNA was isolated from a region of carcinoma and a region of adenoma contained within the same tumor specimen. The larger of the two polymorphic alleles at the pYNZ22 locus in each case is designated as "1", and the smaller is designated as "2". Larger alleles contain more copies of the tandem repeat sequences present in the pYNZ22 probe, and therefore hybridize more strongly to the probe than do smaller alleles. In lane 1C, no loss of either allele was seen in the carcinoma; however, each case in lanes 2C to 12C demonstrates the loss of one allele in the DNA prepared from cryostat sections of carcinoma. In lanes 9C to 11C, the carcinoma specimens appear to have two copies of the retained allele. This conclusion was substantiated by rehybridization of the blots to polymorphic probes from other chromosomes to control for DNA loading. Virtually complete loss of the hybridization signal for the deleted allele was observed in some cases (for example, panels 2, 6, 8, and 10). In the other cases (panels 5, 9, and 11) histological analysis of the cryostat sections from tumor regions used to prepare the DNA revealed proportions of nonneoplastic cells (10 to 30%) consistent with the signal remaining in the band representing the lost allele. DNA preparation, Southern transfer, and hybridization analysis were performed as described in the legend to Fig. 1. The DNA probe used for hybridization analysis was the 1.7-kb Bam HI fragment of pYNZ22 (*16*).

Table 1. Clonal loss and gain of chromosomal sequences in colorectal carcinomas. DNA from normal colonic mucosa and cryostat sections of carcinoma was studied with polymorphic probes from the chromosomes indicated. The number of cases in which the normal tissue was found to be heterozygous for at least one of the probes for each chromosome is indicated as the number of informative cases. The number of informative cases in which loss of one allele was seen in carcinoma tissue is indicated. In a subset of these cases, the loss of one allele was associated with the duplication of the retained allele; the subset of cases that had such loss and duplication events (those homozygous for the marker in the carcinoma DNA, rather than hemizygous) is indicated. Southern hybridizations, as described in the legend to Fig. 1, were performed with the following DNA probes and restriction digests to identify clonal loss or gain or sequences: pYNZ2 (chromosome 1, Taq I digest) (*16*); pλg3 (chromosome 7, Hinf I digest) (*24*); insulin (chromosome 11p, Hinf I digest) (*25*); c-Ha-*ras* (pEJ) (chromosome 11p, Msp I digest) (*26*); p7F12, p9D11, p9A7, p1E8 (chromosome 13q, Msp I digests) (*27*); p79-2-23 (chromosome 16q, Taq I digest) (*28*); pYNZ22 (chromosome 17p, Bam HI digest) (*16*); myosin heavy chain (chromosome 17p, Hind III digest) (*29*); growth hormone (chromosome 17q, Msp I digest) (*30*); thymidine kinase (chromosome 17q, Taq I digest) (*31*); pTHH59 (chromosome 17q, Taq I digest) (*16*); DOSLC 2 (chromosome 20, Msp I digest) (*32*). Each blot was hybridized to probes from several different chromosomes to control for variability in DNA loading.

Cases	Number of cases with loss and gain on chromosome:							
	1	7	11	13	16	17p	17q	20
Informative*	23	28	25	29	27	33	31	10
With losses	2	3	3	0	1	25	8‡	0
With loss of one allele and duplication of retained allele†	0	3	3	0	0	7	3	0
With gains	0	8	5	4	2	0	0	5

*A total of 34 carcinomas was examined. †The cases with loss of one allele and duplication of the retained allele are a subset of the cases with losses. ‡All eight cases with loss of a 17q allele had also lost a 17p allele.

cell, but only that one cell [or a small number of cells which had the same X chromosome inactivated (*20a*)] outgrew its companions during the process of neoplasia. However, our studies demonstrated that even very small adenomas (3 to 4 mm in diameter) were monoclonal, suggesting that this process of selective outgrowth must begin during the early stages of neoplasia.

On the basis of the data presented here and elsewhere, one can begin to formulate a hypothesis for the development of colorectal neoplasia. Early in the neoplastic process, one colonic cell appears to outgrow its companions to form a small, benign neoplasm. The step promoting this outgrowth is currently unknown, but the cells that form these benign neoplasms have undergone a generalized alteration in their genomes, that is, DNA hypomethylation (*21*). This change is interesting in that hypomethylation has been shown to inhibit chromosome condensation and may lead to mitotic nondisjunction (*22*), resulting in the loss or gain of chromosomes. During the growth of the adenomatous cells, a mutation in a *ras* gene (usually codon 12 of the c-Ki-*ras* gene) often occurs (*23*). Finally, a loss of tumor suppressor genes on chromosome 17p or, less frequently, on other chromosomes, may be associated with the progression from adenoma to carcinoma. This genetic model of neoplastic development, although rudimentary, is consistent with the multistep nature of human malignancy and can be tested in a variety of experimental systems.

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7. DNA was prepared from cryostat sections of tumor specimens as follows: Tumor specimens were frozen soon after surgical removal and maintained at −80°C until analysis. Fragments of the tumors were embedded in O.C.T. compound (Miles Scientific) and a cryostat section stained with hematoxylin and eosin (H&E) was prepared for microscopic examination. After examining this section, either (i) regions of the tumor specimen containing high proportions (75 to 95%) of neoplastic cells were removed from the frozen block with a scalpel and re-embedded in O.C.T. or (ii) regions containing non-neoplastic cells were removed from the frozen block with a scalpel. A total of 103 6-μm sections were then prepared. Sections 1, 52, and 103 were stained with H&E for histologic analysis (*8*); the remaining 100 sections were pooled and DNA was purified from them as described by S. E. Goelz, S. R. Hamilton, and B. Vogelstein [*Biochem. Biophys. Res. Commun.* **130**, 118 (1985)].
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17. Of the 30 adenomas studied, 15 were obtained from three patients with familial adenomatous polyposis and 15 were spontaneous adenomas from nine patients without familial adenomatous polyposis.
18. The one adenoma with a clonal loss of chromosome 17p markers was of villous type, had areas of high-grade dysplasia, and was obtained from a patient with familial adenomatous polyposis. Although only one of the 30 adenomas was found to have a clonal loss (that is, loss of one allele in greater than 90% of the neoplastic cells), a 30 to 50% decrease in the intensity of one allele was seen in each of two spontaneous adenomas. Histopathological analysis of the cryostat sections demonstrated that the regions of adenoma used to prepare DNA contained greater than 90% neoplastic cells in both cases. This observation, therefore, suggested that only a fraction of the neoplastic cells in these two adenomas had lost chromosome 17p sequences.
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Cloning and Detection of DNA from a Nonculturable Plant Pathogenic Mycoplasma-like Organism

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The ability to detect, quantify, and differentiate nonculturable mycoplasma-like organisms (MLOs) would greatly facilitate epidemiological and taxonomical studies of this unique group of plant and insect pathogens. DNA isolated from extracts of insects infected with the Western X-disease MLO was cloned in *Escherichia coli*. X-disease–specific clones, when labeled and used as probes, readily detected X-disease MLOs in infected plants and insects but did not hybridize with DNA from healthy plants or insects, or from several other plant pathogenic MLOs or spiroplasmas. These methods provide both a sensitive diagnostic tool and a basis for genetically differentiating MLOs.

ALTHOUGH MYCOPLASMA-LIKE ORganisms (MLOs) are thought to cause disease in more than 200 species of higher plants (*1*, *2*), the number of disease agents involved has remained a mystery. This confusion has resulted from the lack of methods for the identification or comparison of these pathogens. MLOs are restricted to phloem tissues and are transmitted from diseased to healthy plants by phloem-feeding insects that also become systemically infected with these organisms (*3*). Although MLOs morphologically resemble the culturable mycoplasmas associated with animals and other hosts (*4*), the principal impediment to their definitive classification as mollicutes has been the inability of workers to culture plant pathogenic MLOs in vitro. For this reason, provisional identification of MLOs has relied almost entirely on biological data such as plant host range, disease symptomatology, and pathogen-vector relationships.

Previously, confirmation that individual plants or insects were infected with an MLO depended on the tedious preparation and examination of sectioned tissues with the electron microscope. Although electron microscopy first revealed MLOs in infected plants and insects (*5*), it cannot be used in field studies to identify plant reservoirs or insect vectors of a specific MLO because plant-infecting MLOs are morphologically indistinguishable (*6*). The lack of techniques that can rapidly and specifically identify MLOs in field-collected plants or insects has significantly hindered epidemiological studies on these important plant diseases. In addition to aiding ecological studies, the development of sensitive and specific assays for differentiating MLOs would also facilitate etiologic, taxonomic, and phylogenetic studies on this recalcitrant group of plant pathogens.

Progress has been made in detecting plant-infecting MLOs by the use of serological assays such as the enzyme-linked immunosorbent assay (ELISA). Polyclonal antisera have been produced against partially purified MLO antigens derived from both infected plants (*7–11*) and insect vectors (*9*, *10*). Although serological assays are more rapid and specific than electron microscopy, most polyclonal antisera produced against MLO-enriched extracts had substantial cross-reactivity with healthy host antigens (*7*, *8*). The problems associated with cross-reacting polyclonal antisera can be overcome by producing monoclonal antibodies against MLO antigens (*2*). However, because these highly specific reagents are presumably directed against a single MLO epitope, their utility for classifying MLOs at the species level may be limited. For example, monoclonal antibodies specific for a New Jersey strain of the aster yellows MLO (*2*) failed to detect MLOs in plants with symptoms of aster yellows from other geographical areas (*11*).

We devised a method for the detection and identification of MLOs in plants and insects that utilizes recombinant DNA tech-

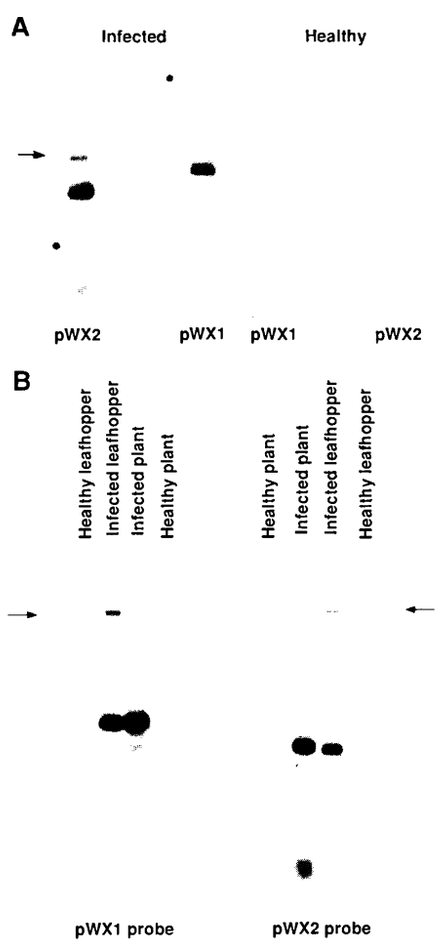

Fig. 1. Southern blot analyses of cloned WX-MLO DNA. (**A**) Plasmids (pWX1 and pWX2) containing WX-MLO sequences were digested with Eco RI and Hind III, electrophoresed in 1% agarose gels, alkali-denatured, transferred to nitrocellulose filters (0.45 μm, BA 85, Schleicher & Schuell) as described by Maniatis *et al.* (*25*) and baked at 80°C for 2 hours. The filters were prehybridized for 6 hours at 42°C in a solution containing 50% formamide, 5× SSPE (0.75*M* NaCl, 50 m*M* Na_2HPO_4, and 5 m*M* EDTA, *p*H 7.6), denatured salmon sperm DNA (200 μg/ml) and 5× Denhardt's solution [0.1% Ficoll, 0.1% polyvinylpyrollidone (M_r 10,000) and 0.1% bovine serum albumin (BSA)]. This solution was replaced with a similar solution containing alkali-denatured, ^{32}P-labeled nick-translated DNA (200 ng; specific activity 7 × 10^7 cpm/μg) from X-diseased or healthy celery. After a 12-hour incubation at 42°C the filters were washed twice (30 minutes each time) in a solution of 2× SSC + 0.1% SDS at 25°C, once in 0.1× SSC + 0.1% SDS at 65°C, and once in 0.1× SSC + 0.1% SDS at 25°C. The filters were dried and exposed to x-ray film (Kodak XAR) for 24 hours with an intensifying screen (Lightening Plus, DuPont) at −70°C. (**B**) DNA from healthy and infected celery and leafhoppers was digested with Eco RI and Hind III and transferred to nitrocellulose filters as described in (A). Nitrocellulose filters were hybridized with ^{32}P-labeled pWX1 or pWX2 and further processed as described in (A). Arrows indicate undigested DNA.

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