Molecular mapping and characterization of two chloroplast-encoded chlorophyll deficient mutants of alfalfa (Medicago sativa L.)
by Donald John Lee

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Crop and Soil Science
Montana State University

Abstract:
A molecular and structural analysis of plastids from two independently isolated cytoplasmically inherited chlorophyll-deficient mutants of alfalfa (Medicaqo sativa L.) was undertaken. This work was done in order to assess the extent of variation in the chloroplast DNA (ctDNA) from both mutants, provide molecular markers for the verification of biparental inheritance of ctDNA in alfalfa, evaluate the developmental state of the mutant plastids and determine the transcriptional and translational differences associated with each mutant phenotype.

Physical maps of chloroplast genomes from phenotypically normal alfalfa and the two mutants were developed and compared. Variation among the ctDNAs mapped in this study was limited to four small scale insertion/deletion or point mutation events that differentiated four distinct chloroplast genotypes, three from normal tissue and one from mutant tissue. While both mutants were observed as somatic sectors, each had a different phenotype; one produced albino tissue and the other yellow-green. Electron microscopy revealed distinct differences in development between the two phenotypes. Plastids from albino tissue had no internal membrane development, sparse stroma with vacuoles and osmiophyllic globuli, and envelope degradation. Plastids from yellow-green tissue had limited thylkoid membrane development, no grana, relatively dense stroma with ribosome-like particles, osmiophyllic globules, and various degrees of vacuolization.

Biparental inheritance of chloroplast DNA in alfalfa was determined by using the unique restriction fragment patterns characterized for the normal and mutant genotypes coupled with the chlorophyll deficient phenotypic marker allowed. Sexual progeny expressing the yellow-green sectored phenotype contained paternal ctDNA in the chlorophyll deficient sectors and maternal ctDNA in the normal sectors. Heteroplasmic cells containing both normal and mutant plastids at various stages of sorting-out were observed by electron microscopy of mesophyll cells in mosaic tissue from hybrid plants. This observation confirmed the biparental transmission of plastids in alfalfa.

The characterization of chlorophyll levels in the yellow-green mutant revealed a 72% reduction in total chlorophyll and a lower chlorophyll a/b ratio relative to normal tissue. This major chlorophyll loss was accompanied by a dramatic reduction of many proteins from yellow-green chloroplasts including coupling factor 1 alpha and beta subunits, photosystem II 32 kd protein (PSII 32kd), and the light-harvesting complex proteins of photosystem I and II. Ribulose bisphosphate large subunit (LSU) and small subunit, however, were found at normal levels in yellow-green chloroplasts. Northern blots detected apparently normal levels of expression of the chloroplast encoded genes for LSU and PSII 32kd. Albino tissue showed a complete loss of plastid proteins in SDS polyacrylamide gels and a total absence of chloroplast rRNA in Northerns. Both mutants are believed to undergo arrested plastid development due to pleiotropic effects of altered plastome encoded genes critical to two distinct stages in chloroplast development.

MOLECULAR MAPPING AND CHARACTERIZATION OF TWO CHLOROPLAST-ENCODED CHLOROPHYLL DEFICIENT MUTANTS OF ALFALFA (*MEDICAGO SATIVA* L.)

by

Donald John Lee

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ABSTRACT

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CHAPTER 1

INTRODUCTION

Chlorophyll deficient mutants of higher plants have traditionally been utilized in genetic studies because of their obvious phenotypes and direct relationship to photosynthesis. The studies reported here focus on the characterization of molecular and structural aspects of two chloroplast encoded chlorophyll deficient mutants of alfalfa (Medicago sativa L.). The purpose of these studies was to test the reported biparental transmission of alfalfa plastids with a molecular analysis of the chloroplast genomes of these mutants and to evaluate mechanisms by which the mutant phenotypes could be realized.

The goals of the first part of this study were to construct physical maps of the chloroplast genomes of the mutants, determine the amount of ctDNA variation among mutant and normal plastid genotypes and compare plastid ultrastructure in mutant and normal phenotypes. The goal of the second part of this study was to utilize the ctDNA variation and mutant plastid morphology characterized in Chapter I to verify biparental plastid inheritance in alfalfa. The final part of this study focused on transcriptional and translational aspects of the two mutants. This was done in order to compare these mutants to others previously studied and to determine the level at which the plastome mutations prohibit normal chloroplast development.

CHAPTER 2

CHLOROPLAST GENOME MAPPING AND PLASTID ULTRASTRUCTURE ANALYSIS OF CHLOROPHYLL DEFICIENT MUTANTS OF ALFALFA

Introduction

The chloroplast genome (ctDNA) of higher plants has been mapped in many species and entirely sequenced in tobacco (Nicotiana tabacum L.) and liverwort (Marchantia polymorpha L.) (Fluhr and Edelman, 1981; Palmer, 1985; Umesono and Ozeki, 1987). These studies demonstrate a high degree of gene order conservation across genera and low rates of intraspecific sequence variation indicating the ctDNA is the most highly conserved eukaryotic genome known (Lamppa and Bendich, 1981; Palmer, 1985). Variation in chloroplast genome arrangement has been utilized to establish and reevaluate phylogenetic and evolutionary relationships in plants (Gordon et al., 1982; Palmer and Jorgensen, 1985; Palmer et al., 1985; Palmer and Zamir, 1982; Shoemaker et al., 1986). Variation within genera and species has been documented but limited to small insertion/deletion events and a low frequency of point mutations (Gordon et al., 1982; Metzlaf et al., 1981; Palmer et al., 1985; Palmer and Zamir, 1985; Teeri et al., 1985; Tsunewaki and Ogihara, 1984). A higher degree of larger scale ctDNA rearrangement has been documented in species lacking the duplicated set of ribosomal RNA and ribosomal protein genes arranged as an inverted repeat sequence (Palmer and Thompson, 1981; Palmer and Thompson, 1982).

Pigment deficient mutants in many plant species are one phenotypic class where intraspecific chloroplast genome variation has been identified (Archer et al., 1987; Gordon et al., 1980; Wong-Staal and Wildman, 1973). Most mutants are a result of nuclear gene mutations (Harpster et al., 1980; Tilney-Bassett, 1984), but some cytoplasmically inherited mutants have been documented (Archer et al., 1987; Borner et al., 1972; Duesing et al., 1985; Gordon et al., 1982; Kirk and Tilney-Basset, 1967; Kutzelnigg and Stubbe, 1974; Palmer and Mascia, 1980; Stubbe and Hermann, 1982; Wildman et al., 1973).

Recently Smith et al. (1986) studied a yellow-green mutant of alfalfa that arose from tissue culture and an albino mutant of alfalfa identified in breeding populations. Genetic and developmental evidence indicated plastome mutations caused these phenotypes and plastids were biparentally inherited. Both yellow-green and albino phenotypes arise as somatic sectors in mosaic tissue. The resulting chimeric plants have normal tissue that apparently sustains the mutant sectors, rendering the mutations non-lethal.

The objectives of this study were to determine differences in the ctDNA and examine plastid ultrastructural features from normal and chlorophyll deficient tissue from these alfalfa mutants. Southern blot analysis was used to generate chloroplast genome maps from yellow-green and albino sectors of chimeric plants, normal green sectors of the same plants and their normal parents. Electron microscopy was utilized to evaluate the developmental stage of mutant and normal plastids from the same plants.

Materials and Methods

Plant Sources

Tissue sources utilized in this study were pure sectors from tetraploid *Medicago sativa* plants expressing either the yellow-green sectoring (YGS, a somaclonal variant) or albino sectoring (ALS, discovered in a breeding population) phenotypes described previously (Smith et al., 1986). All designations used followed those established for the plants and tissue provided by E.T. Bingham or S.E. Smith. The plants used were crosses of the original YGS and ALS plants to 6-4, a standard male-sterile tester genotype. An additional mapping analysis was performed on crosses of YGS to MAG, a second normal genotype with a multifoliate morphological trait. These chimeric plants had the pedigree [MAG x YGS] and [(MAG x YGS) x MAG] (denoted 32B-2). All plants were grown in the greenhouse under 16 hour daylength.

Total and Chloroplast DNA Extractions

Pure yellow-green, albino or normal sectors were removed from plants and freeze-dried in a VirTis freezedryer for 2-4 days. Total DNA was extracted from freeze-dried material using the method of Saghai-Maroof et al. (1984). Chloroplast DNA was extracted from fresh tissue using methods described by Kemble (1987).

Restriction Endonuclease Digestion, Gel Electrophoresis

DNA samples were digested with Hind III, Bam H1, Xho I and Pst I either singly or in combination according to suppliers' instructions (IBI or BRL).

Approximately 10ug of digested total DNA or 5ug of ctDNA per lane were loaded onto a 0.7%, 110 x 135 mm agarose gel and electrophoresis was carried out on a BRL horizontal apparatus at 1 V/cm for 16 hours in TBE gel buffer (Maniatas et al., 1982). Gels were then stained with ethidium bromide and photographed under UV light. Mobilities of the Lambda Hind III markers were measured from the photograph.

Southern Transfers

Transfer of restriction fragments from agarose gels to Zeta-Probe nylon membranes or nitrocellulose filters was performed according to the methods of Reed (1985) and Southern (1975) respectively. Nitrocellulose filters were baked at 80 degrees C. in a vacuum oven for 2 hours after transfer was completed.

Hybridization Probes

A library of alfalfa ctDNA PstI fragments cloned into pBR322 and transformed into DH_2B or RR1 E. coli cell lines served as hybridization probes to the filter bound DNA fragments. This library, developed by Dr. J.D. Palmer, covers the entire alfalfa chloroplast genome except for a 35 kilobase (kb) PstI fragment (Fig. 1). Three mung bean Sal I or Sal I/Pst I ctDNA fragments were ligated into either pBR322 or pUC18 that completely span this 35 kb region and complete this library (pers. comm., J.D. Palmer). Plasmids were isolated from E. coli hosts using the mini-prep procedure of Birboim and Doly (1979). Mini-prep DNA extractions were digested with Pst I and run on a 0.7% agarose minigel to verify insert size. DNA from minipreps was labeled with ^{32}P dNTPs using nick-translation (Rigby et al., 1977). Unincorporated labeled nucleotides were separated from labeled recombinant probes using

centrifuged sephadex G-50 1 cc columns. Prior to hybridization, the labeled probes were denatured in solution with 200 mls of 0.2 N NaOH by heating to 100 degrees C for 10 min. Salmon sperm DNA was added to the mix prior to heating.

Hybridizations

Zeta probe and nitrocellulose filters were prehybridized in 25-30 mls of a 1.5 X SSPE, 1.0% SDS and 0.5% Blotto solution or a 50% formamide, 5x Denhardt's solution, 5x SSPE and 0.1% SDS solution. Filters were prehybridized at 67 degrees C. in an water incubator with gentle shaking for 4-24 hours. Denatured probes were added to the bagged filters and prehybridization mix and the bags were resealed and incubated at 67 C for 24 hours.

Washing and Autoradiography

The hybridization mixture containing probe was recovered and reused in up to four hybridizations. Three washes at room temperature with 300 to 500 mls of 2 X SSC/ 0.1% SDS, 0.5 X SSC/ 0.1% SDS and 0.1 X SSC/ 0.1% SDS solutions successively were followed by one wash with 300 to 500 mls of 0.1 X SSC/ 1% SDS at 55-60 degrees.

Washed filters were wrapped in Saran wrap and taped to a Kodak X-ray exposure cassette with one intensifying screen. Autoradiography was performed at -70 C for 2-72 hours. To improve resolution, some autoradiographs were exposed at room temperature without the use of intensifying screens.

Filters were reused by washing in 1000 mls of 0.1 X SSC/ 0.5% SDS at 95 C for 30-60 min. to remove hybridization probe. Prehybridization and hybridization procedures were then repeated.

Fragment Size Determination

Sizes of hybridized filter bound fragments detected by autoradiography were determined by a regression analysis method described by Schaffer and Sederoff (1981). Gels showing nonuniformity in lambda HindIII fragment mobility were subdivided so that unknown fragment sizes were determined from regressions of markers no further than 4 lanes distant.

Electron Microscopy

Ultrastructural features of mesophyll cells from pure sectors of normal, yellow-green or albino leaf tissue were examined by transmission electron microscopy. The tissue was prepared for ultrathin sectioning largely according to the procedure of Carroll and Mayhew (1976). Finely chopped leaves were fixed in 3.0% glutaraldehyde in 0.01 m potassium phosphate buffer, pH 7.2, for 24 hours. Fixation in 2.0% osmium tetroxide in the same phosphate buffer followed. Tissue dehydration was in a graded concentration series of ethanol (50-100%). Ethanol was replaced with propylene oxide before infiltration of the fixed tissue with Spurr's embedment. Polymerized tissue was then thick sectioned for cytological observation under the light microscope. Selected regions of embedded tissue were thin sectioned on an ultramicrotome, mounted on grids and stained with uranyl acetate and lead citrate. Grids were then observed under the Zeiss EM 10C transmission electron microscope.

Results

Genome Sizes

Chloroplast genome sizes for all genotypes analyzed were in agreement with alfalfa chloroplast genome sizes determined by Rose et al (1986) and Palmer (personal communication). Number and sizes of fragments generated by each of the restriction endonucleases mapped are listed in Table 1.

Comparative Blots

Since total DNA from both mutant and normal tissue was used in the Southern analysis to determine restriction maps, comparative blots of chloroplast and total DNA from the normal tissue in 6-4, MAG, 6-4 x YGS and 6-4 x ALS plants were performed. With all probes, bands representing homology of the ctDNA with mitochondrial or nuclear sequences were easily distinguished from chloroplast DNA fragments. All strongly hybridized bands present in total DNA were present in ctDNA (data not shown).

Polymorphisms

Polymorphisms among mutant and normal chloroplast genomes were found with four of the alfalfa ctDNA probes, 2,3,6 and 7 (Figure 1). These Pst I fragment probes reveal restriction pattern differences with Hind III and Bam HI digests that define three unique normal ctDNA genotypes and one mutant ctDNA genotype.

Polymorphisms with Bam HI restriction fragments hybridized by Pst I probe 7 indicate the loss of a recognition site in both mutants, MAG

Table 1. Alfalfa chloroplast genome fragments produced by restriction endonuclease digestion.

Filter-bound ctDNA Fragments

Probes	Tissue Source	Pst I	Xho I	Hind III	Ban HI
1	all	6.2[1]	5.3,9.0	4.0,3.0,2.1,20.0	2.3,2.1,13.0
2	a	21.0	9.0,6.8,2.9,19.1	20.0,2.6	13.0,1.4,1.5,2.2,2.4,5.5
	b,c,d	"	"	20.0,2.7	13.0,1.4,1.5,2.2,2.4,5.6
3	a	21.0,35.0	19.1,33.5	2.6,5.8	13.0 1.4,1.4,2.2,2.4,5.5,3.3
	b,c,d	"	"	2.7,5.8	13.0,1.4,1.5,2.2,2.4,5.6,3.3
4	all	35.0	33.5	5.8,7.2,9.6	5.0,4.2,23.8
5	all	35.0	33.5	9.6,1.7,2.2,11.5	23.8
6	a,c	12.7	33.5,4.9	11.5,1.4,7.7	23.8..6..7,1.6,1.9,2.8,2.2
	b	12.6	33.4,4.9	11.4,1.4,7.7	23.8..6..7,1.6,1.8,2.8,2.2
	d	12.5	33.3,4.4	11.3,1.4,7.7	23.8..6..7,1.6,1.7,2.8,2.2
7	a	18.0	4.9,3.5,16.8	7.7,1.8,4.4,1.4,2.1,11.5	3.9..9,7.6,1.5,1.6,11.0
	b,c	"	"	7.7,1.8,2.8,1.6,1.4,2.1,11.5	3.9..9,9.1,1.6,11.0
	d	"	"	7.7,1.8,2.8,1.6,1.4,2,1,11.5	3.9..9,7.6,1.5,1.6,11.0
8	all	5.1	16.8	11.5	11.0
9	all	7.0	16.7	11.5,1.4	11.0,2.9,3.4
10	all	7.1	16.7	13.1	3.4,5.0
11	all	12.5	16.7,3.6,1.2,5.3	13.1.5.5,4.0	5.0,3.4,6.5,1.7,2.3
Number of Fragments	a	9	12	21	30
	b,c	"	"	22	29
	d	"	"	22	30
Total Size (Kb)		124.6	128.6	119.3	125.9

[1] all sizes in Kb

tissue sources

a- 6-4, 6-4 x ALS normal, 6-4 x YGS normal

b- yellow-green and albino

c- 32B-2 normal and MAG

d- MAG x YGS normal

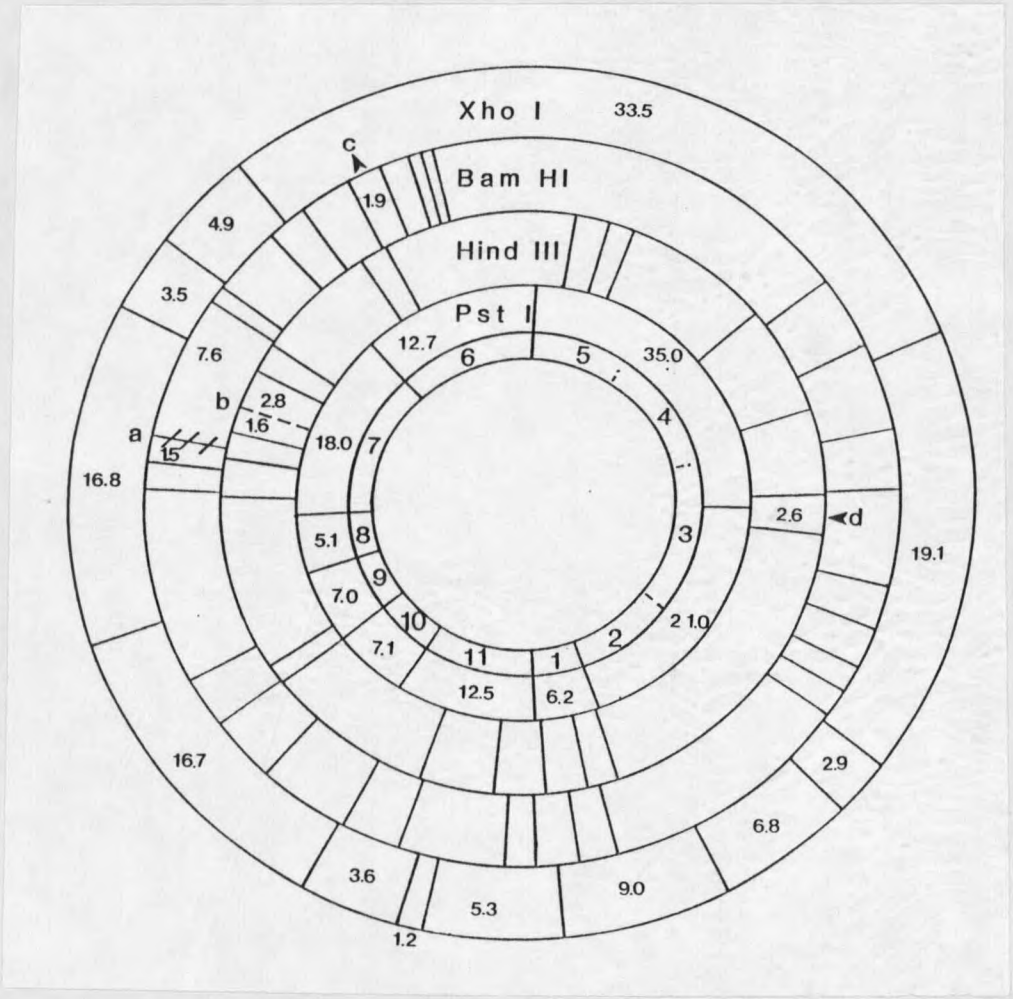


Figure 1. Physical map of the alfalfa chloroplast genome for Pst I, Xho I, Bam HI and Hind III restriction endonucleases as generated from data on Table 1. and all combinations of Bam HI, Hind III and XhoI double digests. Numbered fragments correspond to hybridization probes used in the mapping process. Fragments 1,2, and 6-11 correspond to alfalfa ctDNA Pst I fragments while fragments 3-5 are mung bean ctDNA fragments that are homologous to the alfalfa Pst I 35 kb fragment and part of the 21 kb fragment. All recognition sites are the same for normal and mutant ctDNAs except as denoted as follows:

a. Bam HI site detected by probe 7 found in the 6-4 and MAG x YGS normal and absent in the mutants, MAG and 32B-2 normal tissue.

b. Hind III site detected by probe 7 found only in the mutants, MAG, MAG x YGS and 32B-2 normal tissue.

c. Small deletions detected by probe 6 in a 1.9 kb Bam HI fragment. Approximately 100 bp deleted in albino and yellow-green tissue and 200 bp deleted in MAG x YGS normal tissue.

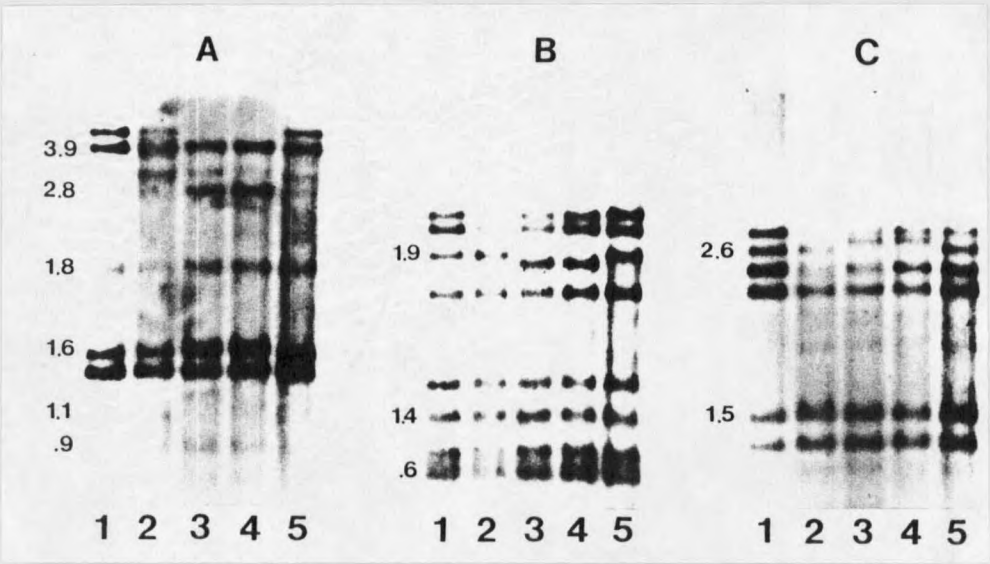
d. 100 bp insertion detected by probes 2 and 3 in the 2.6 kb Hind III fragment found in the mutant, MAG, MAG x YGS, normal and 32B-2 normal tissue.

and 32B-2 normal sector ctDNA (Figure 1). This results in a 9.1 kb fragment in the mutants and a 7.6 plus a 1.5 kb fragment in the normal tissues (Table 1). This point mutation in the Bam HI recognition site is visualized on autoradiographs (Figures 3A and 3B) of Bam HI-Xho I digested fragments hybridized with probe 7. A 6.5 kb fragment in albino, yellow-green, and 32B-2 normal lanes is cleaved to 5.0 and 1.5 kb fragments in lanes containing 6-4, 6-4 x ALS normal sector, and MAG x YGS normal sector DNA. MAG ctDNA restriction fragment patterns have been compared to 6-4 and mutant ctDNAs in a subsequent study (Chapter 3).

Hind III digests show polymorphisms when hybridized by probe 7 as a result of an extra recognition site in the ctDNAs from albino and yellow-green sectors and in normal sectors from MAG x YGS and 32B-2 (Figure 1). The 6-4 ctDNA contains a 4.4 Kb fragment that consists of a 2.8 and a 1.6 Kb fragment in the two mutants (Table 1). Autoradiographs of Bam HI-Hind III double digests hybridized with probe 7 (Figure 2A) demonstrate the Hind III point mutation observed in this region. MAG, MAG x YGS normal sector and 32B-2 normal sector ctDNA have the same Hind III sites as the mutant ctDNAs in this region (data not shown).

Polymorphisms observed by Pst I fragment 2,3 and 6 hybridizations are found with all restriction endonucleases and are a result of addition/deletions rather than point mutations (Figure 1). Since the differences in fragment sizes between mutant and normals is relatively small (100 base pairs) resolution of these polymorphisms is only obvious in smaller fragments. Hybridizations with probe 6 reveal a 100 base

Figure 2. Autoradiographs of Bam HI-Hind III digest fragments hybridized by probes 7,6, and 2. Lanes 1-5 contain the following samples of total DNA (approx. 5 ug per lane); (1) 6-4, (2) 6-4 x YGS normal, (3) 6-4 x YGS mutant, (4) 6-4 x ALS mutant, (5) 6-4 x ALS normal. Sizes of hybridized fragments were determined by regression with lambda Hind III mobilities and are noted in kb on the left of each autoradiograph.

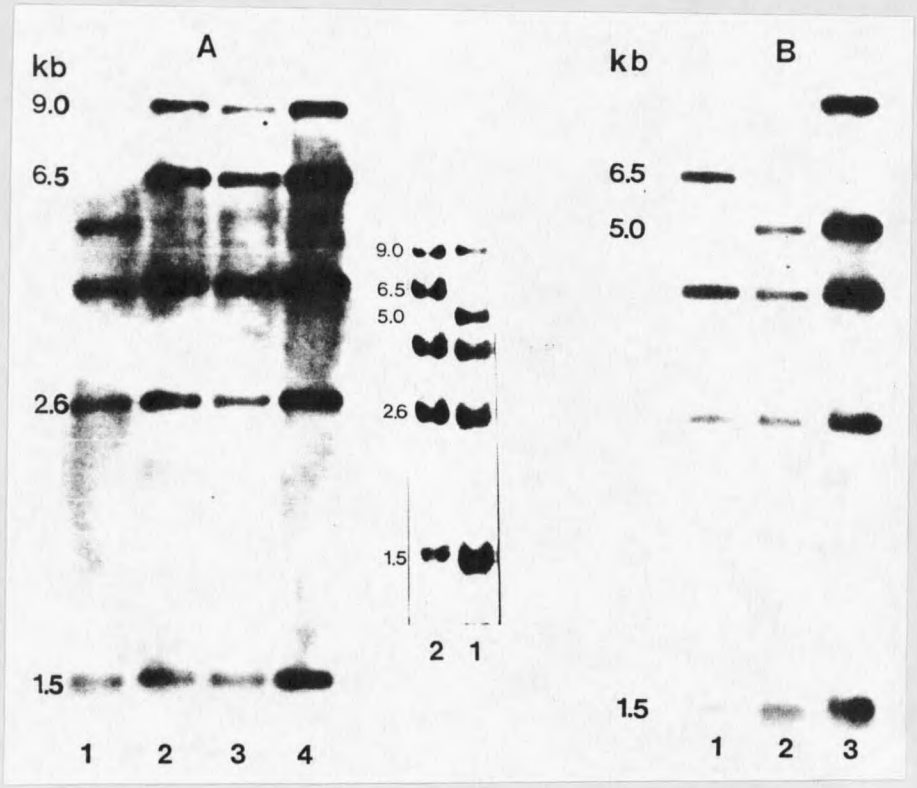


A: Probe 7 reveals a point mutation with Hind III restriction endonuclease. A 4.4 kb Hind III fragment contains an extra Hind III site in the mutants and is cleaved to a 2.8 kb and a 1.6 kb fragment (lanes 3 and 4).

B: Probe 6 hybridized with a 1.9 kb fragment (lanes 1,2 and 5) that is slightly smaller in the mutants (lanes 3 and 4).

C: Probe 2 hybridizes with a 2.6 kb fragment in the normal and 6-4 lanes (1,2 and 5) that is slightly larger in the mutants (lanes 3 and 4).

Figure 3. Autoradiographs demonstrating polymorphisms in normal and mutant ctDNA genotypes. All lanes contain approximately 10 ug of total DNA or 5 ug of ctDNA.



A: Probe 7 hybridized to Bam HI-Xho I digested filter-bound fragments. Lanes contain the following samples of total DNA; (1) MAG x YGS normal, (2) MAG x YGS yellow-green, (3) 32B-2 normal, (4) 32B-2 yellow-green. A 6.5 kb double digest fragment in lanes 2-4 is cleaved to 5.0 and 1.5 kb fragments because of an extra Bam HI site. The extra 1.5 kb fragment is visualized more clearly in a second filter containing lane 1 and 2 samples shown in the inset.

B: Probe 7 hybridized to Bam HI-Xho I fragments. Lanes contain the following total DNA samples; (1) 6-4 x ALS albino tissue, (2) 6-4 x ALS normal tissue, (3) 6-4. Bam HI restriction site gain in lanes 2 and 3 results in a 6.5 kb fragment cleaved to a 5.0 kb and an extra 1.5 kb fragment.