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Isolation of the Menkes disease and mottled genes which encode copper transporting ATPhases

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Isolation of the Menkes disease and Mottled genes which encode copper transporting ATPases

by

Christopher D. Vulpe

DISSERTATION

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Acknowledgments

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It is with profound sadness that I end my stay in graduate school in the laboratories of Jane Gitschier and Seymour Packman. This period has been among the most exciting and interesting times of my life. From the moment I stumbled into their respective laboratories, I have been blessed with their continual support and encouragement.

With Sy I remember vividly our first exciting meeting of possibilities and feasibility when we discussed the project to identify the Menkes disease gene. Since I joined his lab, I have been continually amazed by his continuos good cheer, positive perspective, and unwavering enthusiasm. I was grateful for his unflagging trust in me and my abilities and forbearance for my occasionally prickly personality. As well, I am indebted to Sy for unfolding the arcane intricacies of copper metabolism in our formal and impromptu discussions.

Jane turned a wild idea into a reality by taking me into her lab. She made feasible an improbable project with her support and by grounding the work in careful and thoughtful science. She was always available to discuss my project or the latest wild idea. Her precise thinking and her ability to ferret out the essential questions has been a wonder and an inspiration. Her reevaluation of my data frequently uncovered work requiring further consideration or opened

up avenues for further research. Jane's unbridled ebullient spirit provided inspiration even when I had none. Her understanding of the frustrations inherent in research, sincere sympathy and cogent suggestions have been invaluable in my sometimes difficult project.

I am also thankful to the members of their laboratories. Susan Whitney in Sy's lab was my lab companion for many years and I was forever amazed at her tolerance for my continually chaotic life in the lab, as well as my often discordant musical tastes. She introduced me to Bluegrass and Birding and her calming spirit provided a soothing counterpoint to my perpetual frenzy. I am thankful to Jane's laboratory for taking me in and making me one of their own. Aida(Aiiiiiiida) Metzenberg and Bruce (Fat Ass) Elder kept my bony butt in line in the lab and were always available for invaluable technical consultation on the intricacies of molecular biology or the mysteries of the computer. I was lucky to have lured Soma Das to the Menkes disease project. Her redoubtable technical skill has made prenatal diagnosis for Menkes disease families possible. Her equanimity and serene presence provided a continual oasis of tranquillity in the lab. To Barbara Levinson, without who none of this would have been possible. It was the luckiest day of my life when she joined the project. The frenetic cloning days blur in my memory to images of B.L. with another exciting result, the first Mc1 cDNA, the methodical intonations of sequence gel analysis, the conclusive Northern blot on the X-ray light box upstairs, the realization. Her skill, her dedication and her scientific sixth sense are truly remarkable and the patients with Menkes disease were lucky that she was on the job.

I would also like to thank my friends. Josh Niclas and Jeff Lefstin in my graduate school class who kept me sane throughout by forcibly removing me

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To Laura, my companion through all of these years, whatever I say will be insufficient. Our continual scientific discussions, ruminations, and speculations about our work provided repeated insights into the work and ways to proceed. Your unending emotional support provided a solid foundation which sustained me through difficult times of my own lability. Every time I think of those first hectic years, I am amazed again at not only your understanding and acceptance of the absurd work hours but also your continued encouragement and assistance. I do not believe this work would have been possible without you, and I thank you for everything.

Isolation of the Menkes disease and Mottled genes which encode copper transporting ATPases

Christopher D. Vulpe

Abstract

Menkes disease is an X-linked disorder of copper metabolism characterized by progressive neurological degeneration and death in early childhood. A translocation with a breakpoint in Xq13 proximal to PGK1 in a female patient with Menkes disease was hypothesized to disrupt the Menkes disease locus. DXS441 and PGK1 were determined to flank the X chromosome breakpoint. Altered Sfi1 and Ksp1 fragments in the genome of the translocation patient indicated the close proximity of the translocation breakpoint to PGK1. Multiple PGK1 YACs were obtained, and two identified which spanned the site of the translocation breakpoint. Alu PCR probes, end probes from the YACs, and ultimately genomic lambda clones narrowed the region to a 1.1 kb fragment which spanned the site of the translocation breakpoint. cDNA library screening with a YAC fragment, exon trapping of genomic lambda clone, and subsequent cDNA walking identified a candidate 8.5 kb cDNA. The candidate gene was not expressed in the translocation patient and quantitative or qualitative changes in expression, as well as genomic deletions of the gene, were noted in multiple other Menkes patients. The cDNA encodes a 1500 amino acid protein predicted to be a P-type cation-transporting ATPase most similar to a bacterial copper-transporting ATPase and which additionally contains six putative metal-binding motifs at the amino terminus. The composite data suggest that the isolated gene represents the Menkes disease locus (MNK). The mouse homologue of the gene was subsequently isolated and alterations in its expression in two alleles of the mottled (Mo) mouse noted, therefore suggesting that the mottled mouse is the murine model of Menkes disease. Additional work in this thesis describe attempts to determine the intracellular location of the Menkes copper transporting ATPase. Multiple polyclonal antisera prepared to the Menkes protein were tested, however a specific antisera was not identified. As alternative approach, a epitope tagged complete cDNA was constructed and expressed in a heterologous cell line although a definitive localization will require further work.

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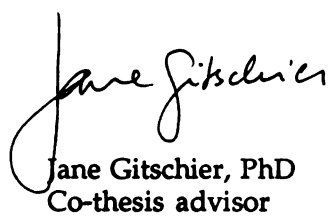
Christopher Vulpe has included two published articles with multiple co-authors in this thesis. In the first, Chris developed all the probes and performed all the physical mapping, isolated the exon-trapped cDNA clones, performed cDNA sequence analysis (together with Barbara Levinson) and assembled the cDNA contigs and analyzed the encoded protein sequence, performed mutation analysis of the Menkes patients by Southern blotting, and wrote the first draft and subsequent drafts of the manuscript in consultation with myself and other members of my laboratory. In the second paper, Chris identified the murine cDNA clones and isolated the 5'-most cDNAs by RACE, sequenced the cDNAs (together with Barbara Levinson), assembled and analyzed the cDNA sequence, and together with the other co-authors prepared the manuscript.



Jane Gitschier, PhD
Co-thesis advisor

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Introduction

Five boys reported from the same extended family with a diverse array of clinical findings including a failure to thrive, peculiar twisted, kinky, friable hair and progressive neurological degeneration including seizures and profound mental retardation and death in early childhood by John H. Menkes in 1962 was the first clinical description of the eponymous Menkes disease (Menkes et al., 1962). Subsequent clinical reports added an additional set of findings including poor temperature regulation, connective tissue defects and decreased skin pigmentation and confirmed the X-linked inheritance pattern (Aguilar et al., 1966; Billings and Degnan, 1971; Bray, 1965; Danks et al., 1971; French et al., 1972; Ghatak et al., 1972; O'Brien and Sampson, 1966; Wesenberg et al., 1969). The distinctive steely or kinky hair in these patients led to its alternative and descriptive name as kinky hair disease. The incidence has been estimated between one in thirty thousand to one in three hundred thousand live births (Danks et al., 1971; Tonnesen et al., 1991).

A distinctive set of pathological findings including prominent cerebral and cerebellar degeneration, as well as connective tissue disease including arterial tortousity and bladder abnormalities are found in patients with Menkes disease (Aguilar et al., 1966; Ghatak et al., 1972; Troost et al., 1982). Unique pathologic findings including cerebellar purkinje cells with somal sprouts (multiple abnormal projections from the nerve cell body) and prominent dendrites often described as having a "weeping willow" appearance are found in this disorder and discriminate it from other causes of neurologic degeneration (Hirano et al., 1977; Matsubara et al., 1978; Purpura et al., 1976; Robain et al., 1988).

The copper connection

The set of disparate finding in patients with Menkes disease was perplexing until 1972 when David Danks in Australia noted that sheep which were starved for the metal copper developed unmarketable steely wool very similar to the hair of patients with Menkes disease (Danks et al., 1972). Copper metabolism was investigated and patients with Menkes disease were found to have severe systemic copper deficiency (Danks et al., 1972; Horn et al., 1975). Copper content is decreased in most tissues including the brain and liver but curiously is increased in other tissues such as the intestine and kidney (Keydorn et al., 1975; Lucky and Hsia, 1979; Nooijen et al., 1981). Cultured cells from patients with Menkes disease, initially fibroblasts (Goka et al., 1976; Horn, 1976) but subsequently many other tissue types (Herd et al., 1987; Vandenberg et al., 1990) with the notable exception of hepatic cells, accumulate copper to abnormally high levels. The cellular phenotype of copper accumulation results in the trapping of copper in the intestine or other tissues of patients with Menkes disease and the failure of copper distribution to the body and subsequent copper deficiency in most tissues in patients with Menkes disease.

The copper deficiency results in decreased activity of copper enzymes in the tissues of patients with Menkes disease (Horn, 1984). Decreased activity of these copper enzymes or function of other copper proteins may result in the clinical phenotype of the affected individuals (Menkes, 1988). For instance deficient activity of the enzyme lysyl oxidase which is involved in collagen cross linking likely contributes to the connective tissue defects seen in these patients (Royce et al., 1980). The decreased pigmentation in the patients with

Menkes disease (Volpintesta, 1974) may result from decreased activity of tyrosinase a copper enzyme involved in melanin synthesis. Mitochondrial cytochrome oxidase deficiency (Sparaco et al., 1993) may underlie the poor temperature regulation in patients with Menkes disease. For some pathologic findings, such as the hair abnormalities, there is no clear connection to known copper proteins and may be due to defects in unidentified copper dependent proteins or to secondary effects.

Defective export of copper is the basic cellular defect in Menkes disease. and leads to the characteristic phenotype of copper accumulation (Garnica et al., 1978). Menkes cells have a specific defect in copper efflux, with normal uptake, and with normal transport of cadmium and zinc in mutant cells (Danks, 1989; Herd et al., 1987). The increased levels of copper induces synthesis of metallothioneins which bind the excess copper in the cytoplasm (Bonewitz and Howell, 1981; Hamer, 1987; Labadie et al., 1981; Sone et al., 1987). As discussed in a subsequent chapter, extensive biochemical work on the nature of the disorder in copper metabolism in Menkes disease suggests a subcellular defect in copper export. However the molecular defect underlying the biochemical abnormalities was not elucidated.

Allelic variants

Clinical variants of Menkes disease with milder or a subset of classic symptoms or with extended survival suggest a wide spectrum of disease could be due to defects in the Menkes disease locus. For example, some mild patients live longer (Sander et al., 1988) or have less neurologic impairment (Danks, 1988; Gerdes et al., 1988; Westman et al., 1988), Patients without connective tissue defects (Haas et al., 1981) and others with disproportionate

connective tissue involvement were identified (Royce and Steinmann, 1990). Mitochondrial myopathy was the presenting symptom in several patients (Inagaki et al., 1988; Morgello et al., 1988). One disorder, variously called X linked cutis laxa (XLCL), occipital horn syndrome, or Ehlers-Danlos syndrome type IX, was initially considered a distinct disease but may represent a mild allelic variant of Menkes disease. Individuals with this disorder have a normal life span and manifest primarily connective tissue disease including lax skin, bladder diverticuli, and bony abnormalities with mild to moderate neurologic impairment in some individuals (Byers et al., 1980; Kuivaniemi et al., 1982). Cell lines from all of these mild and XLCL patients have a defect in copper metabolism indistinguishable from Menkes fibroblasts (Kuivaniemi et al., 1982; Peltonen et al., 1983).

The Mottled mouse

The Mottled mouse represents a mouse model of Menkes disease. The first two alleles of the X-linked Mottled *(Mo)* locus were described in 1953 (Fraser et al., 1953) and since then at least fifteen alleles of the locus were identified (Davisson et al., 1990). Mottled mice exhibit a remarkable similarity to Menkes disease in disease presentation and pathologic findings (Davisson et al., 1990). Considerable phenotypic variability exists among the eight most well characterized alleles. Mo^{pew} (pewter) hemizygous males have mainly hair pigmentation defects while Mo^{dp} (dappled) males die in utero. The two well characterized alleles Mo^{br} (brindled) and Mo^{blo} (blotchy) illustrate the qualitative as well as quantitative differences between the alleles. Mo^{blo} hemizygous males has paled fur, kinked vibrissae, reduced fertility and connective tissue defects often leading to blood vessel lesions and rupture.

Mo^{br} males have no hair pigment, curved vibrissae and hair, and neurologic symptoms manifested in a sustained tremor, a tendency to hind leg clasping and general inactivity with death 14 days post-utero. In many ways, the contrast between *Mo*^{br} and *Mo*^{blo} parallels that between Menkes and X-linked cutis laxa. Connective tissue defects predominate in *Mo*^{blo} and X-linked cutis laxa while *Mo*^{br} and Menkes disease manifest additional neurological symptoms.

Mottled mice exhibit a defect in copper metabolism similar to patients with Menkes disease. D.M. Hunt first noted decreased copper levels and decreased activity of copper enzymes in the tissues of several *Mo* alleles (Hunt, 1974). Subsequently it was demonstrated that Mottled mice cells in culture have a cellular phenotype of copper accumulation (Starcher et al., 1978) due to decreased copper efflux identical to Menkes cells (Camakaris et al., 1980; Packman, 1987; Packman et al., 1984; Packman and O'Toole, 1984; Packman et al., 1983; Packman et al., 1987; Waldrop and Ettinger, 1990).

Copper treatment

Copper treatment is effective in ameliorating the symptoms in Mottled mice of some alleles (Hunt, 1976), but until recently not in patients with Menkes disease. Copper injection between postnatal day 7-14 of *Mo*^{br} (Fujii et al., 1990; Mann et al., 1979; Nagara et al., 1981)and *Mo*^{ml} (macular) (Kawasaki et al., 1988) affected males prevents neurological degeneration and perinatal death observed in untreated mice. In contrast copper treatment of *Mo*^{blo} males has no effect on the connective tissue disease in these mice (Mann et al., 1981). At the inception of the work described in this thesis, there was no evidence of effective copper treatment of Menkes disease. Recently, a

few reports on copper treatment initiated at birth or on patients born prematurely indicate that early copper treatment can ameliorate or prevent the neurologic degeneration although severe connective tissue defects remain (Danks, 1994). Copper treatment initiated later in infancy does not appear to alter the course of the disease (Danks, 1989; Menkes, 1988).

Genetics

The Menkes disease locus was localized on the X chromosome by both linkage studies and comparative mapping of the mouse Mottled locus on the mouse X chromosome. Linkage of the Menkes gene to the X centromere was demonstrated in a study of four families (Horn et al., 1984). RFLP analysis of a polymorphism on the proximal short arm supported linkage to the centromere (Wieacker et al., 1983; Wienker et al., 1983). On the murine X chromosome, the Mo locus is located near the structural gene for phosphoglycerate kinase (Pgk-1) (Brockdorff et al., 1991). The human PGK-1 locus is located in the Xq13, suggesting localization of the human Menkes gene in Xq13 based on homology with the mouse X chromosome (Horn et al., 1984). The identification of female patient with Menkes syndrome and a de novo X:2 reciprocal translocation with an Xq13 breakpoint provided further support for a Xq13 location (Kapur et al., 1987) (Figure 1).

Menkes patient with translocation

We hypothesized that the translocation had disrupted the Menkes disease locus and could provide the means to identify the Menkes disease gene. There was no family history of Menkes disease in the family. X-inactivation studies of the patient indicated that the translocation X chromosomes were active and the normal X chromosome inactive in her cells. Skewed inactivation is generally present in X-autosome translocations and results in a

effectively hemizygous individual with expression from only one X-chromosome. Disruption of the Menkes disease locus by the translocation would therefore not be compensated by the expression of the normal X chromosome in some cells. X-autosome translocations has facilitated the identification of other X linked disorders. We therefore decided to localize and identify the site of the translocation breakpoint on the X chromosome in order to isolate the Menkes disease locus.

I will describe in this thesis the work to identify the Menkes disease locus, its mouse homolog, the Mottled disease locus, and preliminary characterization of the protein product of the Menkes disease locus. In the first chapter, I describe the work to physically map the site of translocation breakpoint in the female patient with Menkes disease, described above, and the identification of alterations in the physical map of her genome indicative of the translocation in the vicinity of the PGK-1 locus using pulsed field gel electrophoresis. In the next chapter, the cloning of the normal genomic region surrounding the PGK-1 locus and the narrowing of the site of the translocation breakpoint to a 1.1 kb region is described. The third chapter covers the identification of the Menkes disease gene and its characterization. The mouse homolog of the Menkes disease and the demonstration of defects in the gene in some Mottled mouse alleles is in the fourth chapter. The construction of a complete Menkes cDNA in a mammalian expression vector and work towards the determination of the subcellular location of the Menkes disease gene product makes up the fifth chapter. Future directions for work and the current status of the field are presented in the final chapter. Cellular copper transport in general is discussed in the Appendix.

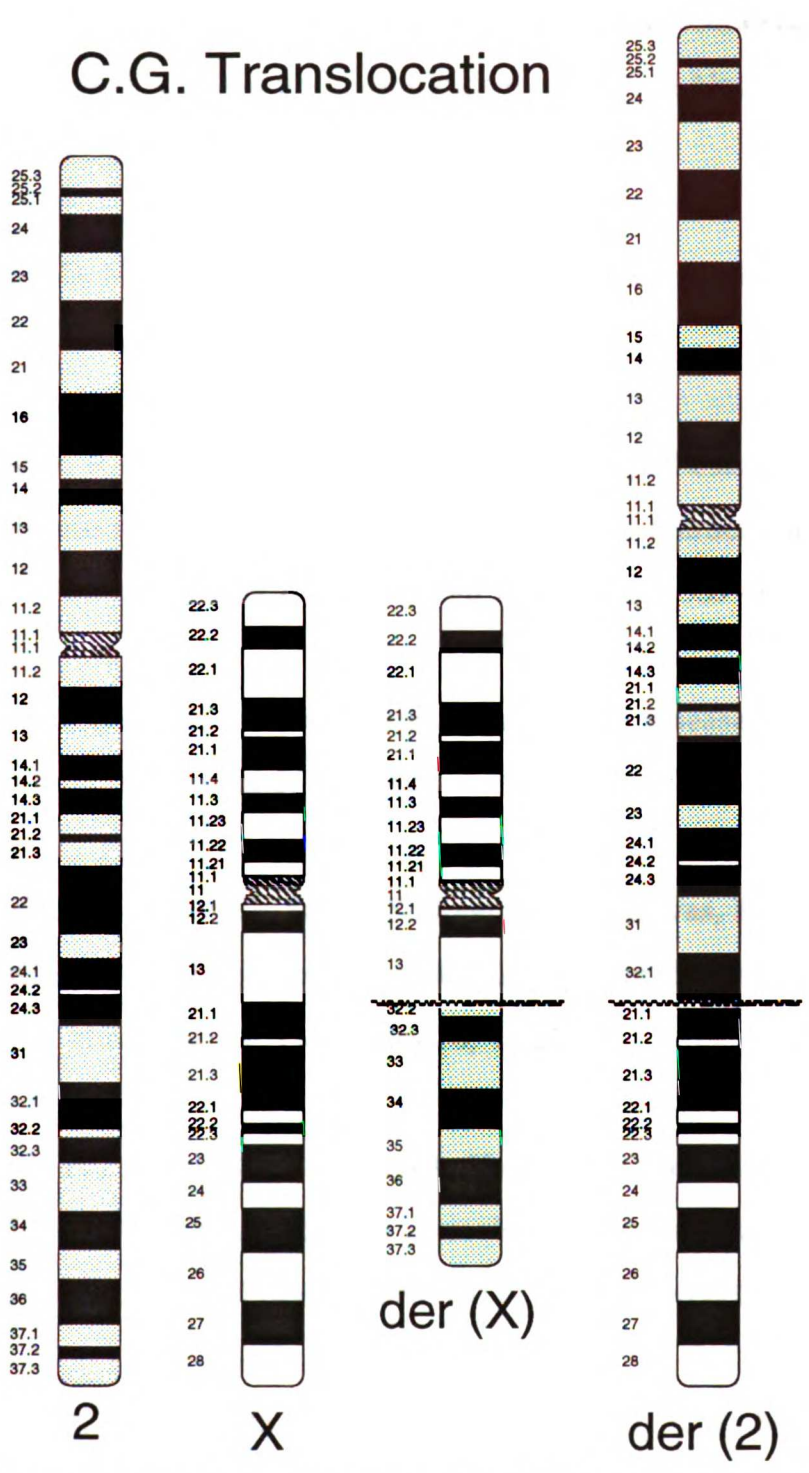

Figure 1: Ideogram of translocation chromosomes in C.G. A female patient with Menkes disease and a reciprocal X:2 translocation with breakpoints in Xq13 and 2q32 is described in the text. The karyotype was confirmed in the cytogenetics laboratory of Dr.Steven Schonberg at UCSF. The normal X was shown to be inactive in the patient. A disruption of the Menkes disease locus by the translocation was hypothesized to be the cause of Menkes disease in the patient C.G.

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Chapter 1

Physical Mapping of the X- chromosome Translocation Breakpoint

Approach

The approach to identify the gene responsible for Menkes disease was based on a female patient who has Menkes disease and also has a balanced X:2 translocation with breakpoints in Xq13 and 2q32.2 (Figure 1). I hypothesized that the translocation had disrupted the Menkes locus. I therefore decided to identify the translocation breakpoint on the X chromosome in this patient as a means to define the chromosomal region where the Menkes locus resides.

Previous work

The site of the translocation breakpoint is proximal to PGK1 in Xq13.3. The der(2) translocation chromosome was separated from the der (X) and normal X chromosomes in somatic cell hybrids by another laboratory (Verga et al 1991). Southern blot analysis with X chromosome probes determined that the breakpoint was in Xq13.3. The PGK1 probe in Xq13.3 and other distal probes were present on the der(2) chromosome. Several other Xq13.3 probes, which had not been ordered relative to each other (Brown et al 1991; Lafreniere et al 1991), were not present on the der(2) chromosome. The site of the X chromosome breakpoint therefore is proximal (centromeric) to PGK1 and telomeric (distal) to the Xq13.3 probes.

Identification of flanking markers

I identified one of those six unordered probes in Xq13.3, DXS441, as the closest proximal marker to the translocation breakpoint, by mapping DXS441 more precisely with a radiation hybrid. The radiation hybrid, 50K1, contains two distinct X chromosome fragments: one including the X centromere and the other the PGK-1 locus (Benham et al 1989). I utilized this radiation hybrid, generously provided by Dr. Goodfellow, to help us determine which

of the six proximal probes were closest to the PGK-1 locus and, by inference, closest to the translocation breakpoint. Southern analysis indicated that 50K1 contained the loci PGK-1, DXS441, and DXS72 (previously mapped to Xq21.1). Additional probes proximal to DXS441 and distal to DXS72 were tested, and these loci were not present in 50K1. A southern blot hybridization of the DXS441 probe present in 50K1 and DXS56, a probe not present, is shown in figure 2. These studies suggest that DXS441 is the proximal (centromeric) marker closest to PGK-1; and suggest that DXS441 and PGK-1 flank the translocation breakpoint on the X chromosome (See Figure 3). Physical mapping of the proband accordingly focused on these probes.

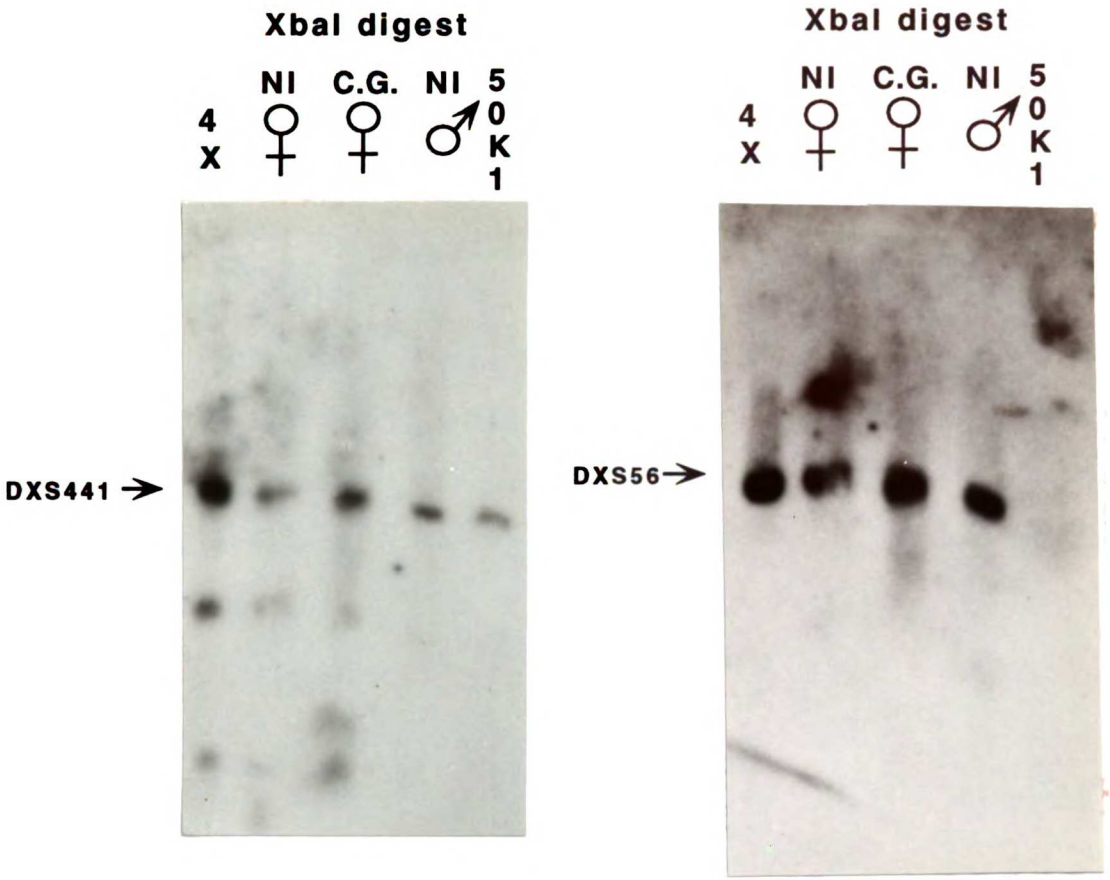

Figure 2: DXS441 is, while DXS56 is not, contained in the 50K1 Radiation Hybrid. The probes DXS441 and DXS56 which had previously been mapped to Xq13, were hybridized to Southern blot of Xbal digested DNA from a control female cell line 4X(GM012012B) with four normal X chromosomes, a normal (NI) control female cell line(GM8447), the C.G. translocation patient, a normal (NI) control male cell line(GM1489) and the radiation hybrid 50K1. 50K1 was characterized previously and shown to contain a piece of genomic DNA surrounding the PGK1 locus. The presence of both PGK1 and DXS441 and the absence of DXS56 in the 50K1 hybrid suggest that DXS441 lies in closer physical proximity to PGK1 than DXS56.

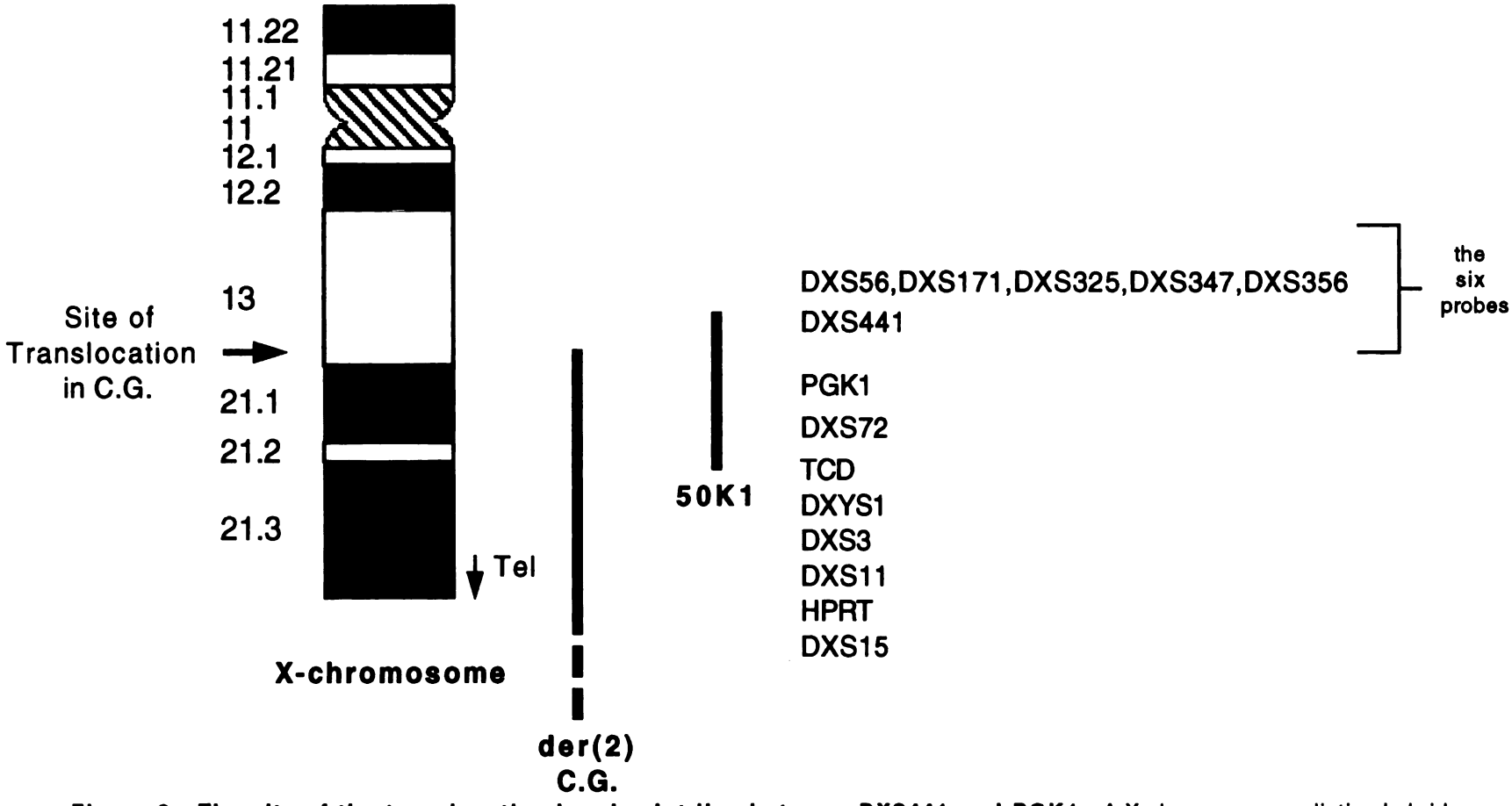

Figure 3: The site of the translocation breakpoint lies between DXS441 and PGK1. A X-chromosome radiation hybrid, 50K1, which contains two small pieces of the X-chromosome in a hamster cell line has been characterized previously and shown to contain the PGK1 locus. Hybridization of previously identified probes in the Xq13 and Xq21 regions to Southern blots of DNA from the 50K1 hybrid demonstrated that 50K1 contains a small genomic region from DXS441(in Xq13) to DXS72(in Xq21) including PGK1. Previous work had shown that the translocation breakpoint in the DNA of the Menkes patient was proximal to PGK1 and distal to the six probes noted in the figure. As DXS441 is the closest proximal probe to PGK1 it is likely the closest proximal probe flanking the translocation breakpoint.

Physical mapping with flanking markers

Pulse field gel elctrophoresis (PFGE) has been a powerful tool in the identification of translocation breakpoints for a number of disorders (Fountain et al 1989; Gessler et al 1989; Merry et al 1990). A translocation is identified by disruption of the physical map on the translocation chromosome in the vicinity of the translocation. Such disruption can result in an altered PFGE fragment which can be detected by a probe in close proximity to a translocation breakpoint (Reily 1989).

Altered PFGE fragments with PGK1 probe

PFGE analysis with the flanking markers found no alteration in the vicinity of DXS441, while alterations were detected with the probe PGK-1. Physical mapping of the proband identified altered PFGE fragments with PGK-1 probes. DNA from the early passage fibroblasts of the proband and from controls was cut and PFGE blots prepared for an extensive series of rare cutter restriction enzymes utilizing separation conditions appropriate to the expected size of the fragments. These blots were subsequently hybridized with the PGK-1 and DXS441 probes. The probe DXS441 detected identical PFGE fragments in the proband and controls for all eighteen enzymes tested, which include AscI, BspCI, BssHII, BsiWI, CspI, EagI, KasI, KspI, MluI, NotI, MluI, PacI, PspA1, SgrA1, SfiI, Sma1, SnaB1, Spo1 and Sse83871. However, altered PFGE fragments were detected with PGK-1 probes in proband DNA cut with SfiI or KspI (SacII isoschizomer). SfiI digestion yields 150kb and 270kb normal bands and a ~700kb altered band (Figure 4). KspI digestion yields a ~300kb fragment in normal controls; in the proband of an additional ~900kb fragment is present (Figure 5). The identification of altered PFGE fragments

in SfiI and KspI digests in the proband suggests the proximity of PGK-1 to the translocation breakpoint on the X chromosome.

Altered PFGE fragments can be due to a number of confounding factors including overloading, partial digestion, methylation differences between tissues and cell lines, and restriction site polymorphisms unrelated to a translocation breakpoint (Antequera et al 1990; Julier & White 1988; Reily 1989; Shmookler Reis & Goldstein 1982). I addressed each of these possibilities in turn. I repeated the digestions multiple times using excess enzyme and loaded equal amounts of DNA for proband and controls; I therefore believe overloading and partial digestion are unlikely explanations for the altered fragments. Furthermore, I utilized the same tissue for all PFGE studies to minimize effects of tissue-specific methylation differences whenever possible. For both enzymes, PFGE analysis found no altered fragments in the DNA from the parents of the proband and from twenty-one normal female controls, therefore suggesting that restriction site polymorphism is an unlikely explanation for the observed PFGE alterations. Additionally somatic cell hybrids (gift of T.Yang) containing either the der(2) or the der(2) and der(X) chromosomes separated from the normal X chromosome contain only the altered fragments and not the normal chromosomal bands.

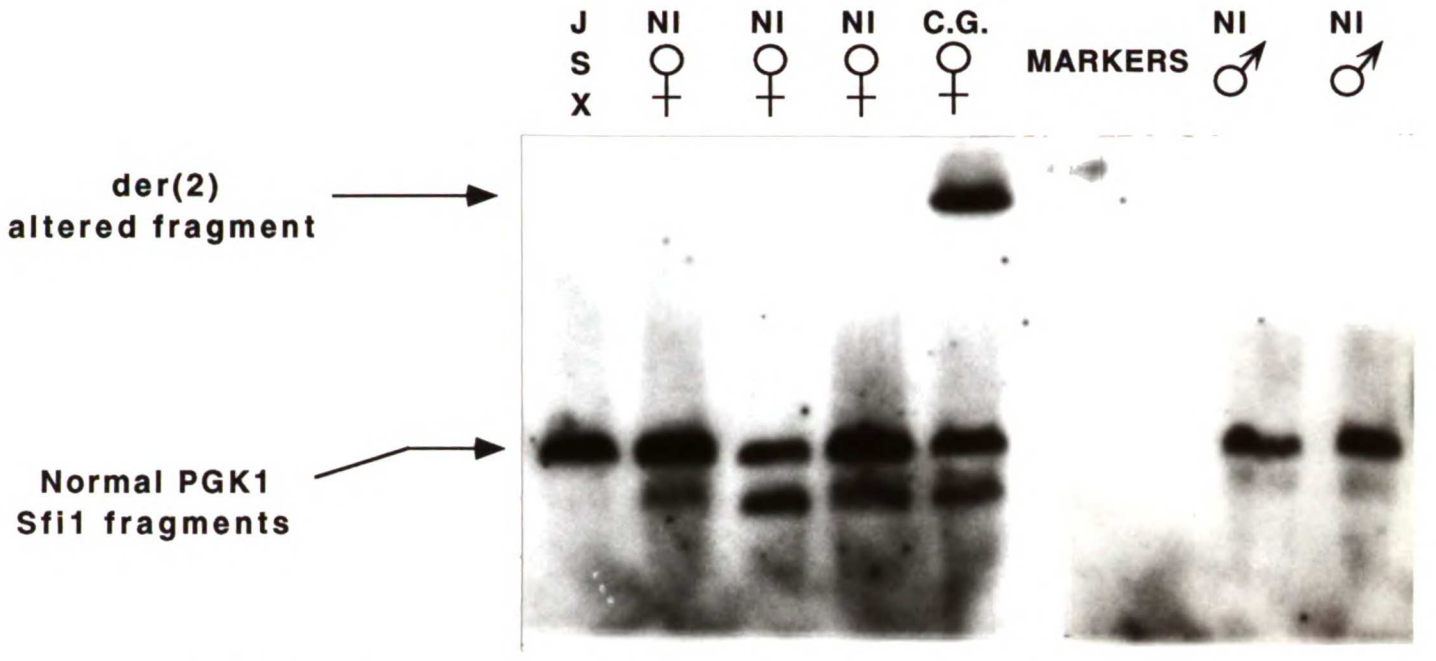

Figure 4: The PGK1 probe detects an altered Sfi1 genomic fragment in the DNA of the patient with Menkes disease and a X:2 reciprocal translocation. JSX represents JSX897B a somatic cell hybrid containing a single X chromosome as it only human material in hamster background. The normal (NI) female cell lines are GM8447, EVE(a 46XX cell line from the Gitschier laboratory) and GM1506. The normal (NI) male cell lines are GM1489 and GM5659. All GM cell lines were obtained from ATCC. Genomic DNA was prepared in agarose blocks, digested for 24 hrs at 50°C in an excess of Sfi1, run on a 1% TBE gel for 36 hrs, 150V, with a 50-90 sec RAMP in a Biorad^{TM} CHEF DRII apparatus. The gel was blotted and probed with a genomic probe, pSPT-PGK1, for the 5' UTR of the PGK1 locus. The PGK1 probe identifies one or two bands of ~150 and ~250 kb in the normal controls. An additional ~700 kb Sfi1 fragment is present in the DNA from C.G. which is not present in normal controls and likely represents a junction fragment between the 2 and X chromosomes in the der(2) chromosome.

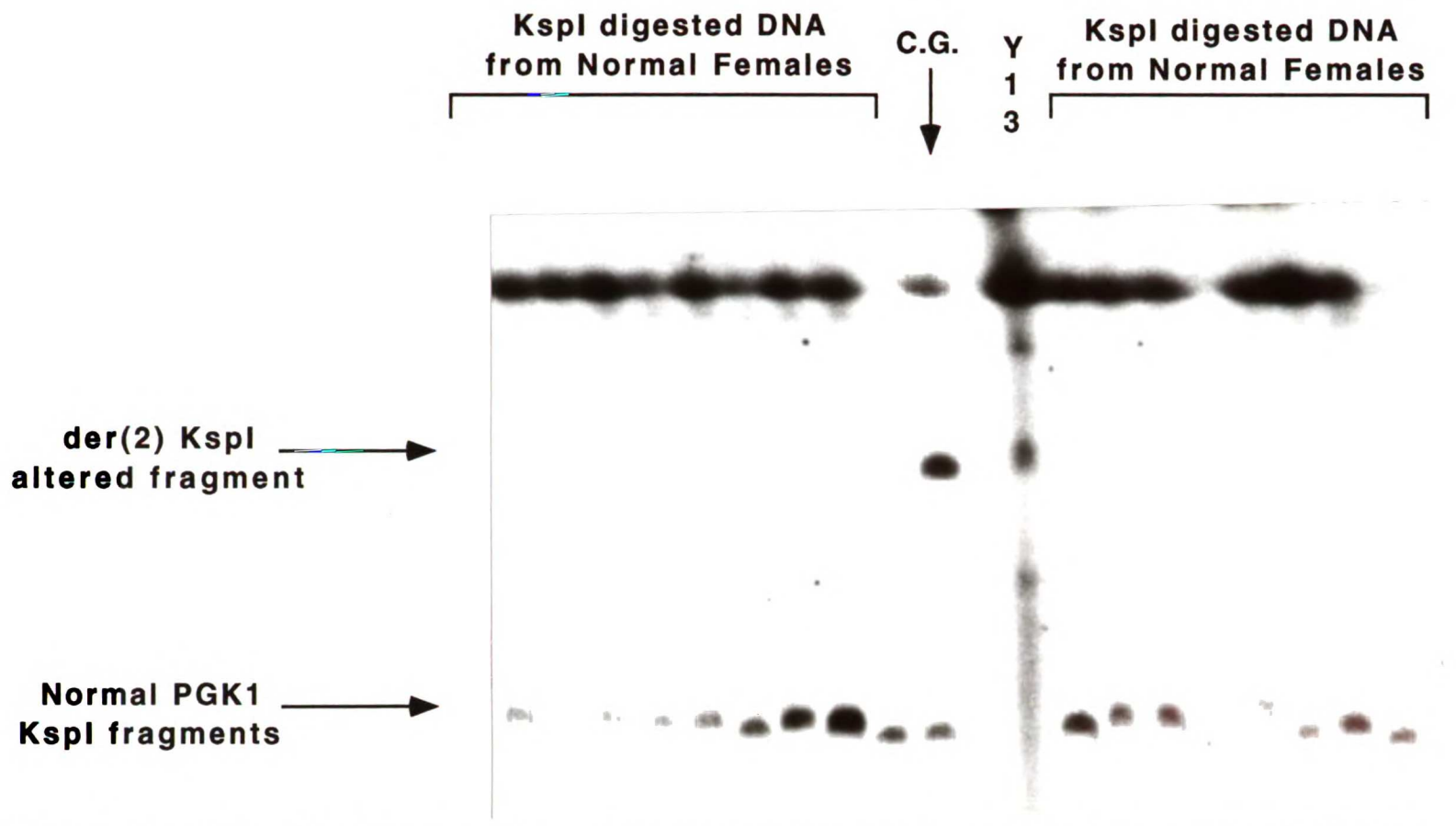

Figure 5: The PGK1 probe detects an altered Ksp1 genomic fragment in the DNA of the patient with Menkes disease and a X:2 reciprocal translocation. Genomic DNA from 17 different normal females, from the translocation patient C.G. and the somatic cell hybrid Y13 which contains the der(2) and der(X) chromosomes were prepared in agarose blocks, digested for 24 hrs at 37°C in an excess of Ksp1, run on a 1.5% TBE gel for 60 hrs, 125 V, with a 50-90 sec RAMP in a Biorad™ CHEF DRII apparatus. The gel was blotted and probed with a genomic probe, pSPT-PGK, for the 5' UTR of the PGK1 locus. The PGK1 probe identifies one or two bands of ~300 kb in the normal controls. An additional ~900 kb SfiI fragment is present in the DNA from C.G. which is not present in normal controls and likely represents a junction fragment between the 2 and X chromosomes in the der(2) chromosome.

Partial digestion physical mapping

Partial digestion physical mapping also supported a causative role of the translocation in the genesis of the alterations. Partial digestion has been used for previous physical mapping studies in other regions (Patterson et al 1987; Rappold & Lehrach 1988). If the altered PFGE fragments were due to polymorphism or methylation, then intentional partial digestion of a normal control should result in the production of a PFGE fragment identical in size to the altered PFGE fragments observed in the proband's DNA. On the other hand, if the altered PFGE fragment is due to the translocation I should not see a fragment of identical size in the partial digest of the normal control. Also, I might expect that partial digestion of the proband may result in novel partial digestion PFGE fragments as compared to the normal control. SfiI partial digestions were performed by increasing NaCl concentration (S.Kenrick and J. Gitschier, pers. comm.) on a normal control and the proband and run on the same gel (Figure 6). The normal control has a strong partial at ~500kb and the next at ~950kb but no partial of the same size as the altered PFGE fragment in the proband. In addition, partial digestion of the proband revealed an additional PFGE fragment of ~800kb not present in the control partial digestions. I suggest that this additional altered PFGE fragment derives from a translocation chromosome and is additional support that the alteration observed is not due to methylation differences or polymorphism. These data suggest a role for the translocation in the genesis of the alterations in PFGE fragment size.

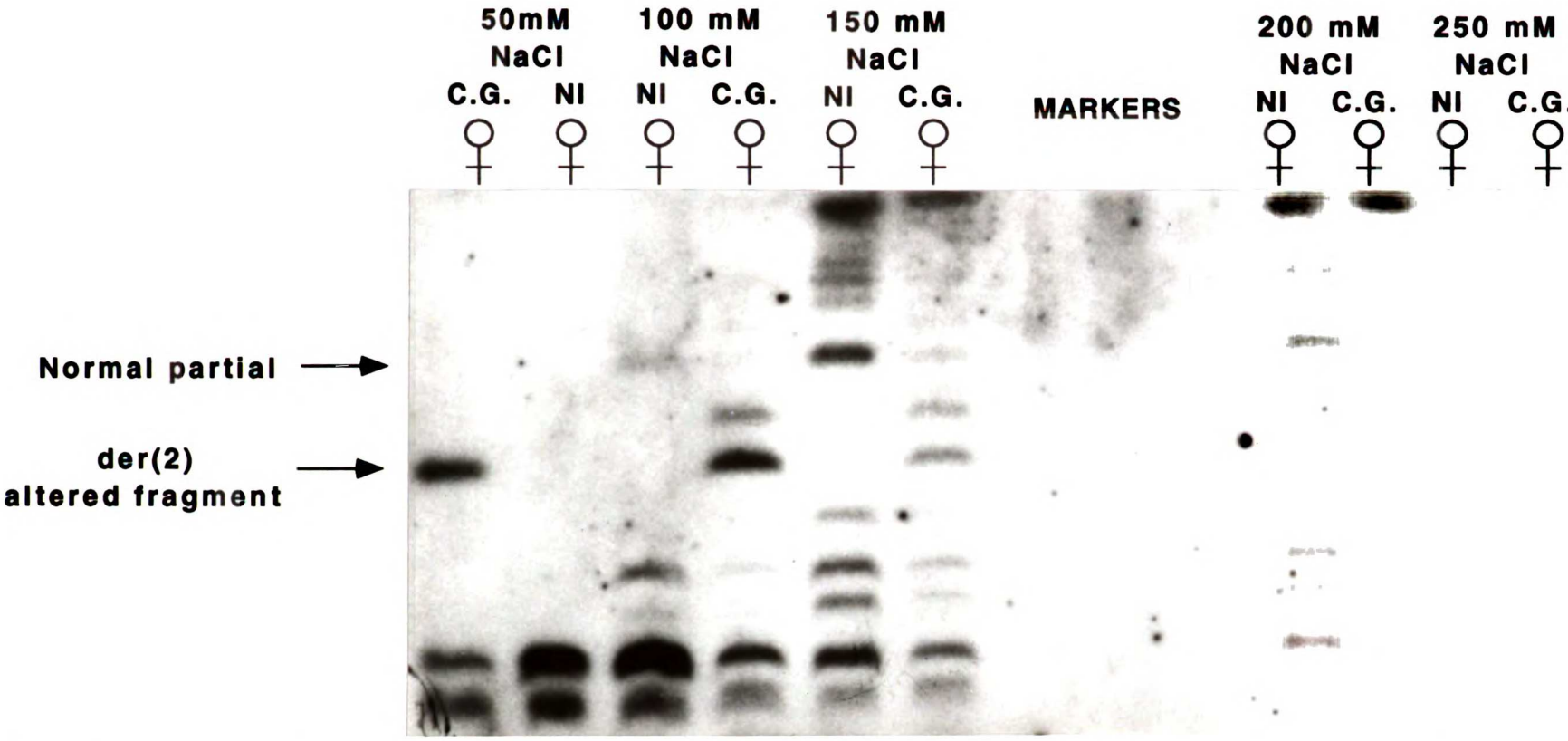

Figure 6: Altered Sfi1 PFGE fragments in DNA from C.G. are not due to partial digestion. Genomic DNA from normal (NI) female cell line GM012012B(with four X chromosomes) and from fibroblasts of the Menkes patient, C.G., with the translocation was digested to completion with forty units of Sfi1, 50° C, 24 hrs in 50mM NaCl buffer(NEB2). Partial Sfi1 digestion(protocol developed by Sue Kenwrick) was with ten units of Sfi1 at 50° C for 3 hrs with increasing NaCl. A 1.5%, 1/2 X TBE gel was run for 60 hrs at 125 V with a 120-180 sec RAMP on a Biorad Chef DRII apparatus and blotted. In the 50mM NaCl lanes above on the left, C.G. DNA is in the first lane and the normal control in the second lane. In the 100mM NaCl lanes and subsequent NaCl concentrations the normal control is first and DNA from C.G. is second. An altered Sfi1 PFGE fragment is present in DNA from C.G. of approximately 700 kb in size. No fragment of the the same size is present in the control DNA at any NaCl concentrations, although a partial digestion product of ~900 kb is present in the normal control(and in C.G. DNA - from the normal X chromosome). These results indicate that the altered PFGE fragment in DNA from C.G. is unlikely due to incomplete digestion or methylation and represents rather a junction fragment between the X chromosome and chromosome 2 in the der(2) chromosome.

It was concluded from the results with the parents, the normal controls, the somatic cell hybrids and the SfiI partials that methylation differences or restriction site polymorphisms are unlikely explanations for the altered PFGE fragments in the proband and therefore it is probable that the translocation plays a causative role. The size of the normal PFGE fragments and SfiI and KspI suggest that the PGK-1 probe is no more than ~300kb from the translocation breakpoint. Therefore I proceeded to physical cloning of DNA around PGK-1 utilizing Yac technology.

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Chapter 2

Identification of the Translocation Breakpoint

Identifcation of PGK1 YACs

Physical mapping studies suggested that the X chromosome translocation breakpoint is within the ~300 kb area between the two KspI sites defined by the PGK1 probe. Seven YACs (Yeast artificial chromosomes) containing the PGK1 locus obtained from three different sources provided genomic DNA in the vicinity of the site of the translocation breakpoint. The YAC 256A identified in the Washington University YAC library was the first YAC identified and spans 130 kb (Brownstein et al., 1989; Burke et al., 1987). Two additional YACs kindly provided by Jennifer Puck from a YAC library prepared from a X-chromosome specific somatic cell hybrid and designated 18H9 and 6E1 were 250 kb and 300 kb in size (Lee et al., 1992). The final source was a ICI laboratories YAC library constructed with DNA from a human cell line containing four X chromosomes and therefore likely enriched for X chromosome clones which at the time of the work had the largest average insert size of YAC clones at approximately 350 kb (Anand et al., 1990). Rakeesh Anand at ICI laboratories kindly performed an initial screen of the YAC library with a PGK1 3' UTR PCR assay (Michelson et al., 1983; Theune et al., 1991) and identified multiple potential positives and initially isolated one YAC 18IA9. This YAC had a physical map very different from previously isolated PGK1 YACs and likely contained a PGK1 pseudogene (Michelson et al., 1985). In order to identify YACs corresponding to the PGK1 locus and not a pseudogene, a PCR assay was developed specific for the PGK1 locus using published PGK1 intron sequence (Michelson et al., 1985). YAC pools positive in the PGK1 3' UTR PCR assay were sent to UCSF. Intron specific PGK1 YAC PCR screening of the YAC pools identified four true positives and four YACs

were subsequently identified, 231F2, 19CH10, 19FC2, 32BA11 of 245 kb, 370 kb, 460 kb, 580 kb respectively.

Physical map of PGK1 YACs

A physical map of the genomic region around PGK1 was constructed utilizing these YACs (Figure 7). As the YACs were obtained, they were tested for the PGK1 locus using a intron specific probe, their size was determined by PFGE analysis and a map was constructed with selected rare cutter restriction enzymes. Analysis initially focused on the enzymes Sfi1 and Ksp1 as altered PFGE fragments were demonstrated in the DNA from the translocation patient with these enzymes with the PGK1 probe. The rare cutter restriction enzyme Eag1 which has a smaller average restriction fragment size than Ksp1 and Sfi1 was utilized subsequently to construct a finer map. Probes corresponding to a PGK1 intron (Smead et al., 1989) and to the non-identical vector sequences in the YAC arms (Burke et al., 1987) which flank the genomic insert of the YAC were used in the initial analysis.

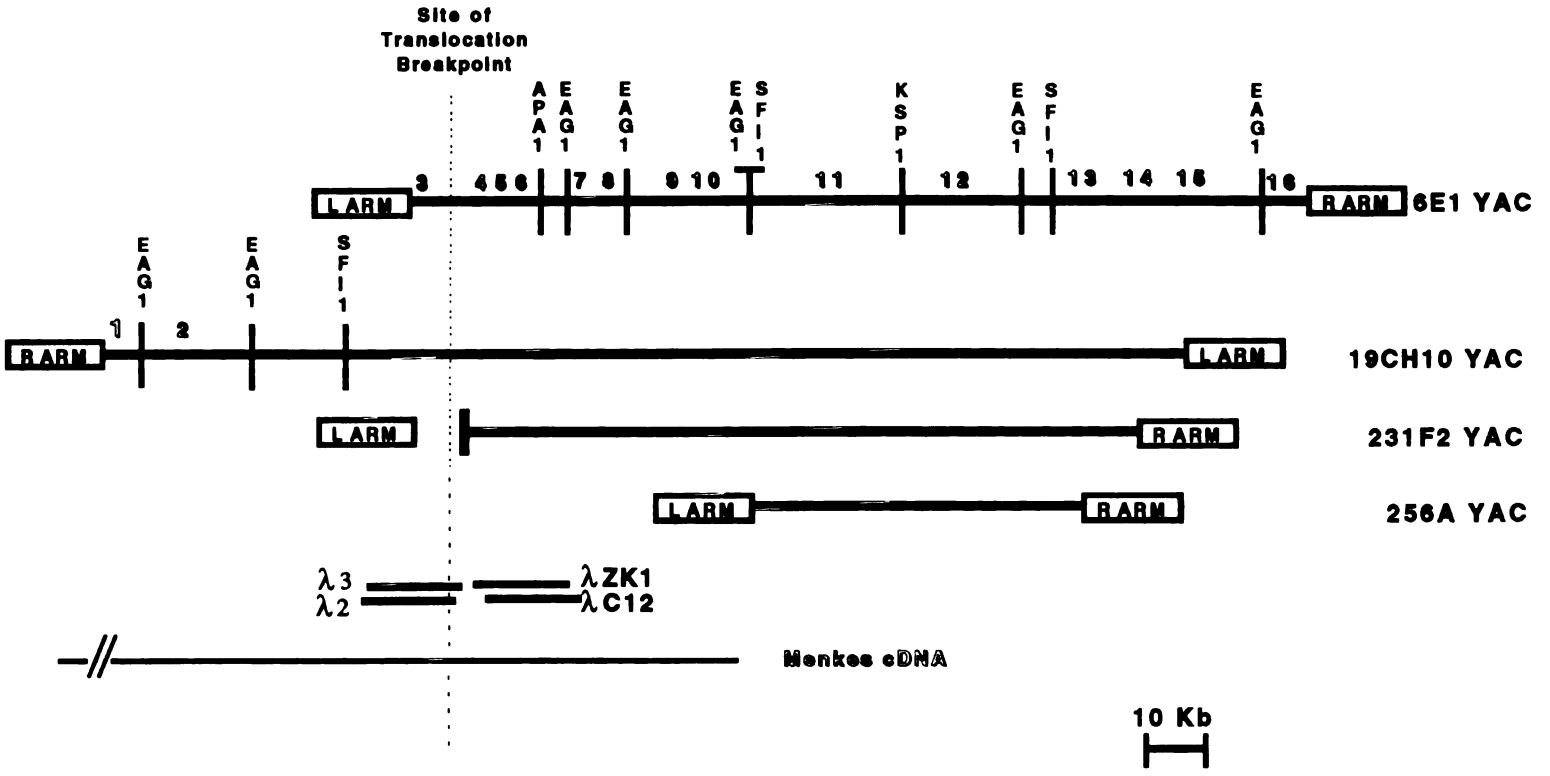
A composite map of selected YACs is presented in Figure 7. Initial characterization of 18H9 indicated that it was a human-hamster chimera, so it was not utilized in constructing the physical map. (A chimeric YAC contains two pieces of DNA from different locations, in this case one human piece containing PGK1 and one hamster piece, juxtaposed in one YAC.) In addition, the yeast containing the YAC 6E1 contained another smaller YAC of hamster origin. Analysis of ICI laboratory YACs focused on the smaller YACs 231F2 and 19CH10 due to a reduced risk of chimerism in smaller YACs, and as described below subsequent developments abrogated the need for analysis of the larger YACs 19FC2 and 32BA11.

Figure 7: Physical map of translocation region. A physical map of the YACs 6E1, 19CH10, 231F2 and 256A was constructed as described in the text by multiple hybridizations of blots of YAC DNA digested with Apa1, Eag1, Sfi1, Ksp1 and combinations of enzymes. Probes are indicated by numbers and the location of the number reflects the approximate location of the probe. The PGK1 probe was an intron specific probe obtained from ATCC, the Alu PCR probes, end probes, and cDNA probes were generated as described in the text. λ 2 and λ 3 were identified from a sorted X chromosome specific library by hybridization to the 6E1 left end probe. The isolation of the Menkes cDNA is described in subsequent chapters and is included here for comparison. I include below a table of PCR primers and conditions used to generate the Alu PCR probes included on the YAC map. The primer sequences are:

TC65- 5'AAGTCGCGGCCGCTTGCAGTGAGCCGAGAT 3'
L1HS -5'AAGTGTCGACCATGGCACATGTATACATATGTAACA(A/T)AACC3'
278 - 5'CCGAATTCGCCTCCCAAAGTGCTGGGATTACAG3'
517 - 5'CGACCTCGAGATCT(C/T)(G/A)GCTCACTGCAA3'
Alu V - 5'CGGGATCCCAAAGTGCTGGGATTACAGGCGTG 3'

PCR product	Primer(s)	Conditions
ZB1	TC65	D-94°C, 1'; A-55°C, 1'; E-72°C, 4' 35 cycles
ZF1B, ZF1T	TC65+L1HS	same
ZG1B	278+L1HS	same
ZK1	517	same
ZN1.1	ALUV	same

PHYSICAL MAP OF TRANSLOCATION REGION



1 - 19CH10 Yac Right End probe
2 - Mc2 cDNA
3 - 6E1 Yac Left End probe
4 - 231F2 Yac Left end probe
5 - ZN1.1 Alu Pcr Probe
6 - ZK1 Alu Pcr probe
7 - ZB1 Alu Pcr Probe
8 - ZF1B Alu Pcr Probe
9 - ZG1B Alu Pcr Probe
10 - ZN1.4 Alu Pcr Probe
11 - PGK1 probe
12 - Mc6 cDNA
13 - 231F2 Yac Right End probe
14 - 19CH10 Yac Left End probe
15 - ZF1T Alu Pcr probe
16 - 6E1 Yac Right End probe

YACs spanning translocation breakpoint

Genomic probes from the ends of the YAC inserts demonstrated that two YAC clones, 6E1 and 19CH10, spanned the translocation breakpoint. End probes were isolated from 231F2, 6E1, and 19CH10 YACs representing the two ends of each genomic insert utilizing the vectorette PCR method (Riley et al., 1990). Briefly, In vectorette PCR the YAC DNA is cut with a restriction enzyme which give small genomic fragments. A special unidirectional linker is ligated to the digested DNA and a genomic end piece is amplified using one primer specific to the linker and the other specific to either the right or left YAC arm. These genomic YAC end pieces are specific for each YAC and differ from the YAC vector arm probes. Probes corresponding to each end of the genomic insert of the YACs, designated right or left end probe depending on the vector end PCR primer utilized, were mapped relative to the translocation utilizing somatic cell hybrids containing the isolated der(2) or the der(2) and der(X) chromosomes. [The human- hamster somatic cell hybrids were the result of a collaboration with T.P. Yang. The C.G. translocation fibroblast cell line was sent to the laboratory of T.P.Yang where hybrids containing the isolated der(2) and der(2) plus der(X) chromosomes were prepared.] As mentioned above, a similar analysis determined that PGK1 is distal (telomeric) to the translocation breakpoint. The 6E1 left end genomic probe and the 19CH10 right arm genomic probe were present in the der(2) and der(X) hybrid and not the der(2) hybrid indicating that they mapped proximal (centromeric) to the breakpoint and that these YACs must span the translocation breakpoint (data not shown). A somatic cell hybrid mapping panel provided by Hunt Willard (Brown et al., 1991; Lafreniere et al., 1991) confirmed that these end probes are located in Xq13 therefore making chimerism an unlikely explanation for the results (Figure 8). And

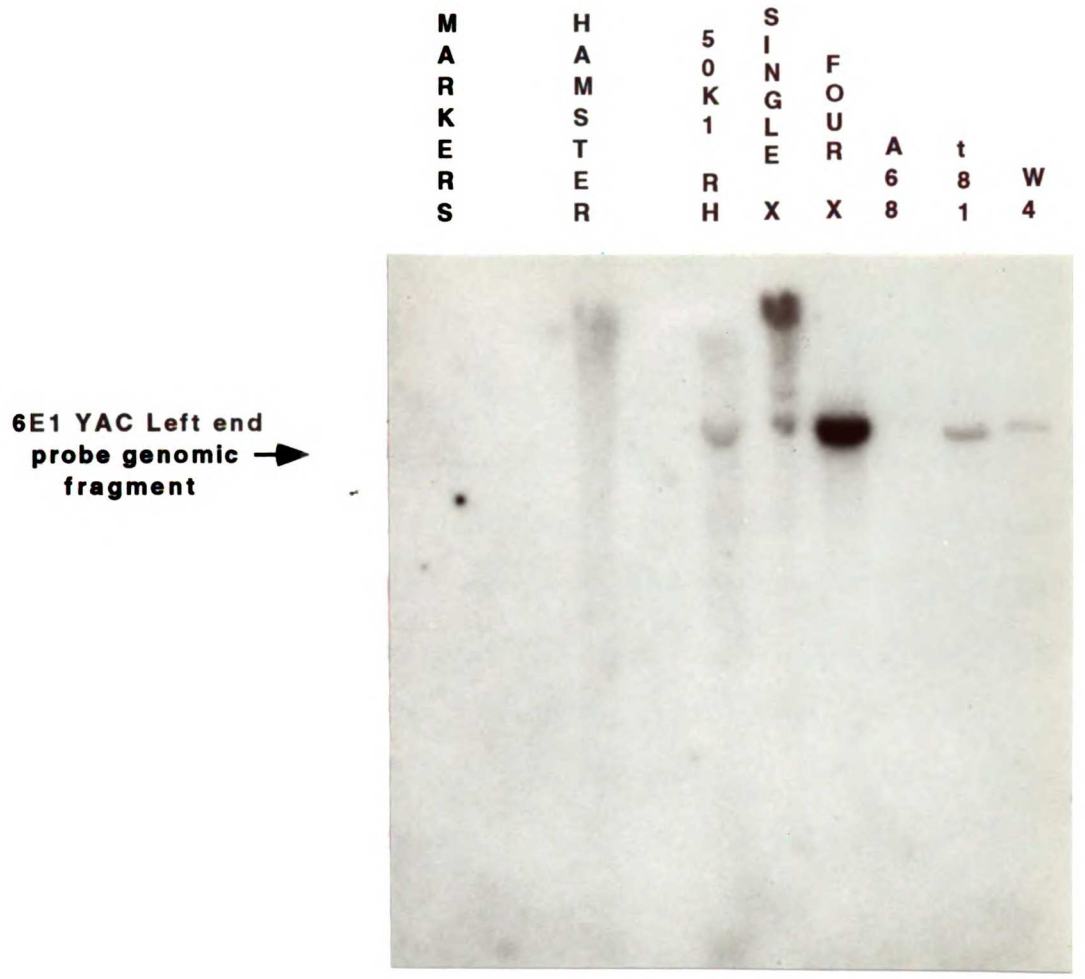


Figure 8: 6E1 YAC left end probe maps to Xq13. Hamster control is GM459. 50K1 is a human X chromosome radiation hybrid in a hamster background which contains a genomic region around the PGK1 locus in Xq13 as described in text. Single X is JSX8973B, a somatic cell hybrid containing a single X chromosome in a hamster background. 4X is GM012012B, a human cell line containing four normal X chromosomes. A68, t81 and W4 are somatic cell mapping hybrids containing various X chromosome translocation chromosomes with breakpoints in the Xq13 region. Ten micrograms of genomic DNA prepared from the cell lines were digested with EcoRI and run on a 1% TAE agarose gel, 50V, for 24 hrs and alkali blotted. A 6E1 YAC left end probe was hybridized to the blot and detected corresponding genomic fragments in all of the cell lines(including A68 although not visible on this exposure) except hamster, indicating that it maps to Xq13. Varying hybridization signal likely reflects different amounts of human DNA present in each cell line.

furthermore, the 6E1 Left end probe hybridized to the same normal Sfi1 and Ksp1 PFGE fragments as the PGK1 probe indicating the probes proximity.

Altered der(X) SfiI fragment

The 6E1 left end probe also detected an additional Sfi1 fragment in the C.G. translocation patient which was not present in normal controls and was distinct from the altered Sfi1 PFGE fragment detected with the PGK1 probe (Figure 9). This altered fragment likely represents the reciprocal der(X) chromosome and provides corroboration that the 6E1 left end probe is on the other side of the site of the translocation breakpoint as PGK1. An additional KSP1 fragment was not detected under the separation conditions utilized and may be smaller or larger than the resolved fragments (data not shown). Over 100 kb lay between the PGK1 probe and the 6E1 left end probe so it was necessary to identify additional probes in the interval to further narrow the site of the translocation breakpoint.

Alu PCR probes

Alu PCR provides probes distributed along the PGK1 YACs. Alu PCR is a method to produce anonymous human probes from complex DNA sources such as a yeast containing a human YAC (Ledbetter et al., 1990; Nelson et al., 1989). Alu is a repetitive element present about every four kilobases in human DNA but not in yeast. Alu specific primers allow the PCR amplification of the DNA between closely spaced Alu repeats in the human genomic DNA in a YAC (Nelson, 1990). Multiple products result from the different PCR amplifications and can represent DNA amplified between different Alu repeats distributed along the length of the YAC. The PCR products can be isolated and represent probes similarly distributed relatively

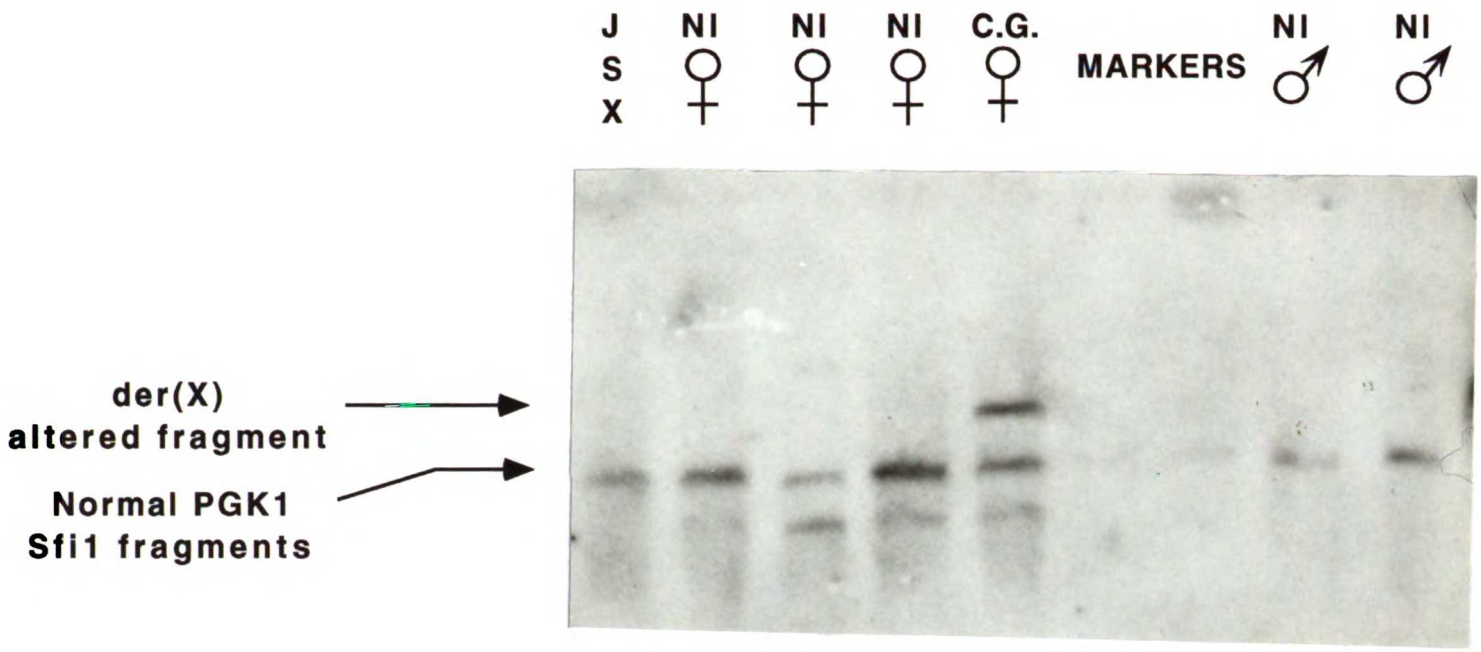


Figure 9: The 6E1 left end probe detects a different altered Sfi1 PFGE fragment than PGK1. The identical blot as described in Figure 4 was hybridized with a probe which contains genomic DNA from the left end of the 6E1 YAC. Sfi1 fragments were detected in normal (NI)controls of the identical size as detected by the PGK1 probe and therefore indicated that the 6E1 left end probe and PGK1 are on the same normal Sfi1 fragment. An additional Sfi1 fragment of ~350 kb is present in DNA from C.G. which is different than the ~700 kb altered fragment detected with PGK1. This additional altered fragment likely represents a Sfi1 fragment spanning the junction of the X and 2 chromosomes in the der(X) chromosome of C.G. The 6E1 Left end probe therefore likely is proximal to breakpoint while PGK1 is distal.

randomly along the genomic DNA contained in the YAC. Alu PCR amplification of the YACs indicated that some Alu PCR products, as assayed by size, were shared by all the PGK1 YACs tested and likely correspond to genomic regions shared by all the YACs, while other products were shared only by a subset or were unique to a particular YAC and likely represent products from genomic regions not shared in common by the YACs. These YAC Alu PCR "fingerprints" (Nelson, 1990) were used to identify probes in the gap between the flanking probes, PGK1 and the 6E1 left end probe. Alu PCR products present in the 6E1 YAC and not present in the 256A YAC likely derive from genomic DNA in this gap or the smaller region distal to PGK1 (away from the breakpoint) not contained in 256A. Multiple different Alu PCR primers were used to this end, and an example of the reaction products for the YACs 256A, 18H9 and 6E1 is presented in Figure 10. As can be seen, there are products present in 6E1 which are not present in 256A (or 18H9) and these were isolated as possible probes in the region of interest. The PCR primers utilized to amplify each probe are presented in legend of Figure 7.

Breakpoint region narrowed to 40 kb

Alu PCR probes and 231F2 left end probe localized the site of the translocation breakpoint within forty kilobases. Each isolated probe was hybridized to panel of the PGK1 YACs and most of the probes isolated in this manner mapped to 6E1 and not 256A. Such 6E1 specific probes were subsequently cloned into a plasmid vector and their location more precisely mapped relative to Eag1, Sfi1 and Ksp1 sites in the YACs. These probes allowed a more detailed restriction map of the YACs to be constructed (Figure 7). End probes corresponding to the ends of the genomic inserts of the YACs

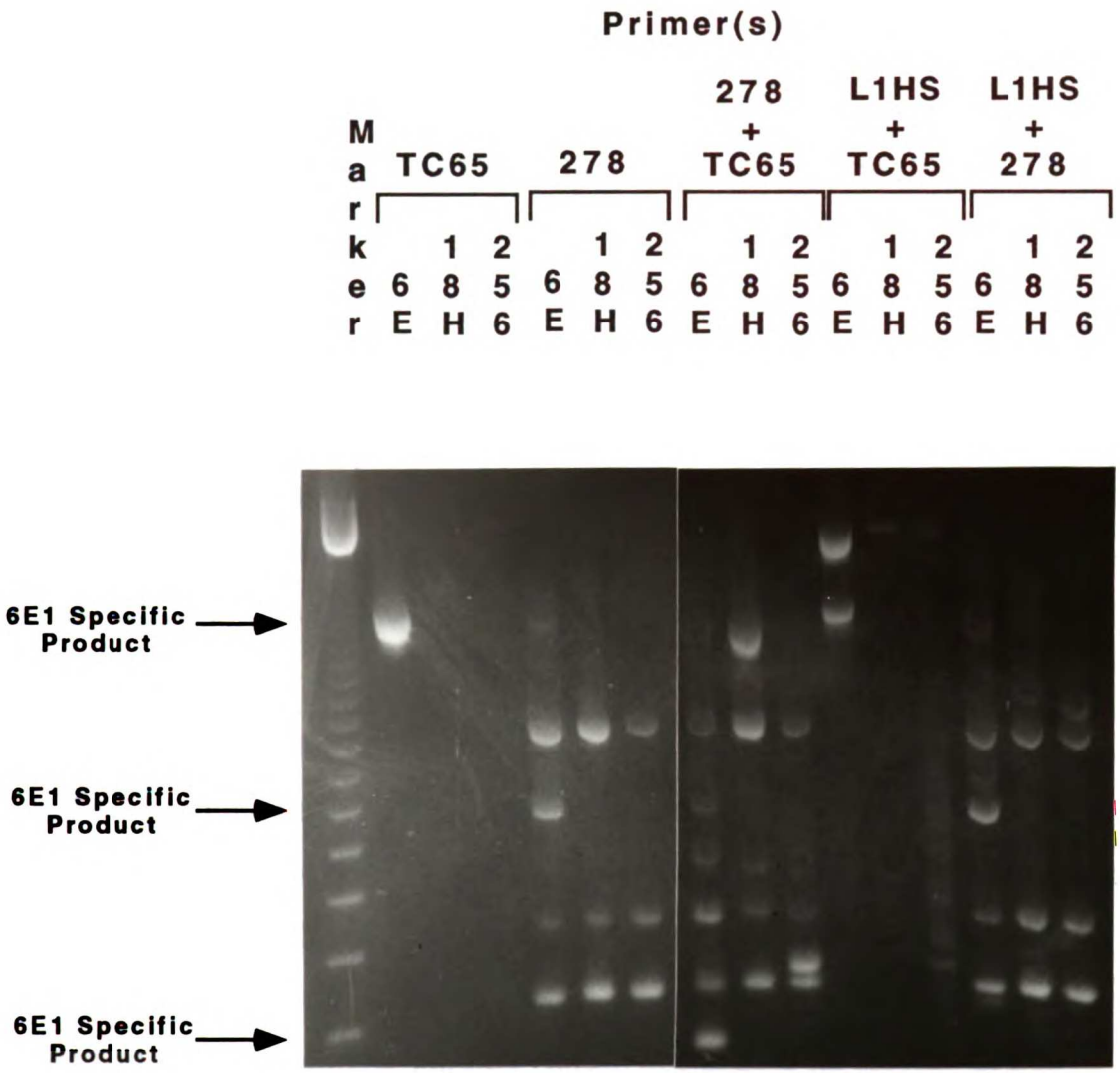


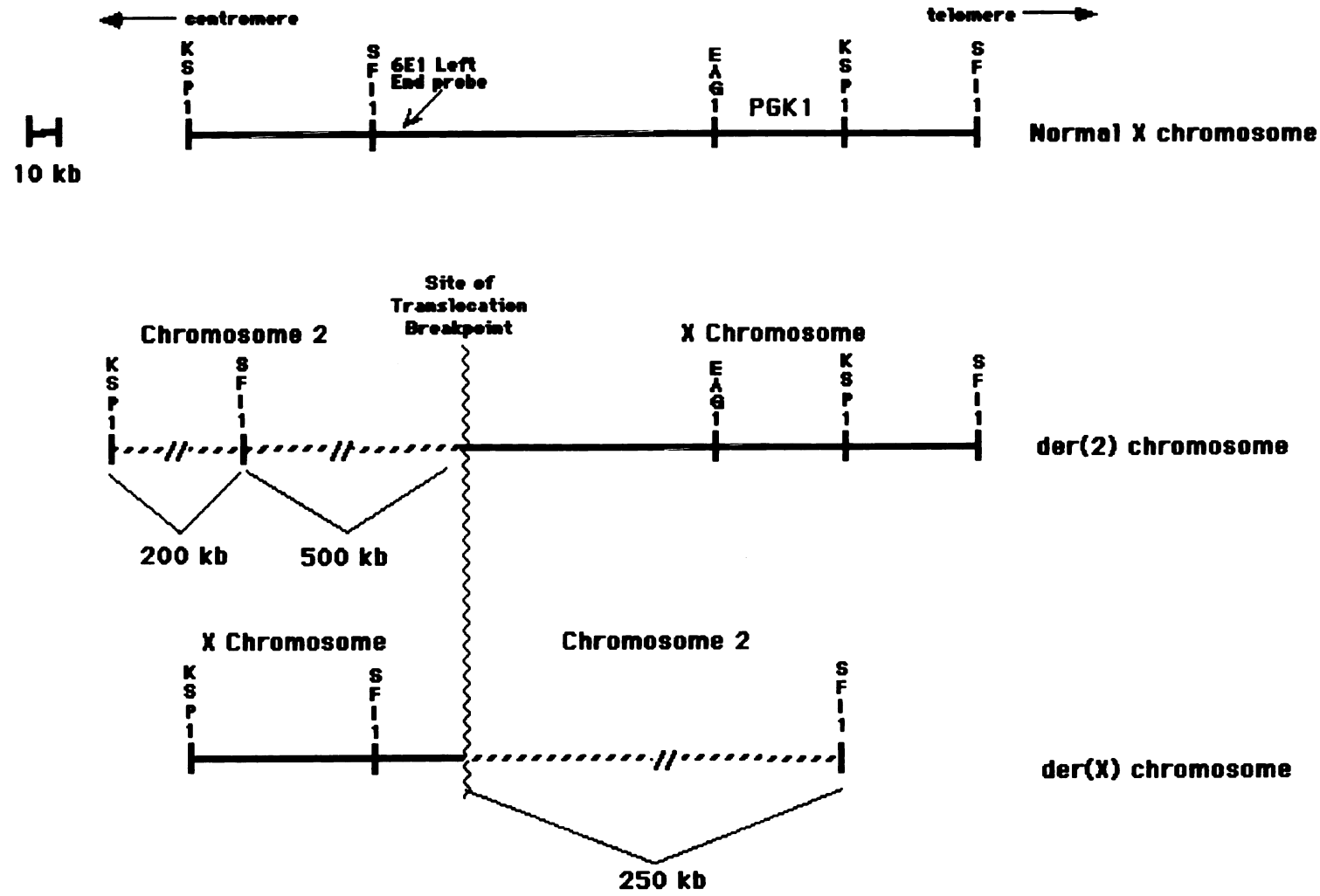
Figure 10: Alu PCR fingerprints identify Alu PCR products specific to YAC 6E1. DNA was prepared from yeast containing the PGK1 YACs, 256A, 18H9, and 6E1. PCR with primers specific to human repetitive elements amplifies DNA between closely spaced elements in the human YACs. TC65 and 278 are oppositely oriented primers in the human Alu element, while L1HS is a primer to the LINE1 element. As can be seen in the first set of lanes, showing amplification products with TC65, a product is present in the 6E1 lane which is not present in the other YACs and likely derives from sequences present in 6E1 which are not present in 18H9 and 256A. Similar representative products with other amplification primers or combinations are indicated by arrows at left. 6E1 specific products were gel purified and used in subsequent mapping studies.

6E1, 19CH10, 231F2 were also mapped onto this composite restriction map. Each probe was also tested for its location relative to the breakpoint utilizing the derivative chromosome hybrids and genomic PFGE analysis of DNA from the translocation patient. A composite genomic PFGE map of the translocation region is presented in Figure 11. All of the Alu PCR probes and YAC end probes, with the exception of the 19CH10 right end and the 6E1 left end probes as described above, mapped distal (the PGK1 side) to the breakpoint (data not shown). Three distal probes, two Alu PCR probes and the 231F2 YAC left end probe, mapped within 40 kb from the proximal 6E1 YAC left end probe and therefore defined a small region containing the site of the translocation breakpoint.

Genomic lambda clone span breakpoint

Two genomic Lambda clones span the site of the translocation breakpoint. The four closest probes flanking the site of the translocation breakpoint were used to screen a X-chromosome specific genomic lambda library (ATCC 57750). Barbara Levinson screened with the proximal 6E1 Left end probe and identified two overlapping lambda clones, designated λ2 and λ3, in this region. The three distal probes identified five overlapping lambda clones. Restriction mapping of the lambda clones, and the hybridization studies with Sst1 subclones prepared from representative Lambda clones indicated that the proximal and distal lambda clones did not overlap. Mapping of each lambda subclone relative to the breakpoint as above revealed that the lambda isolated with the three distal probes did not cross the site of the breakpoint. Subclones of λ2 and λ3, however, mapped to both sides of the translocation breakpoint and suggested that the site of the translocation breakpoint must lie within the lambda clones (Figure 7). Subsequent fluorescent in situ genomic

Genomic physical map around the site of the translocation breakpoint



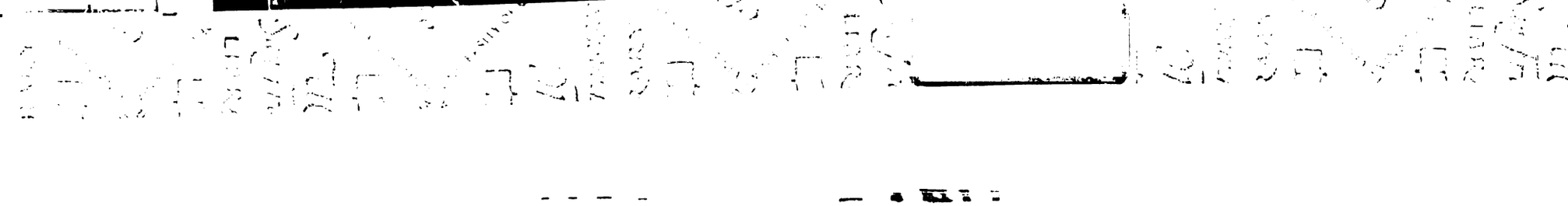


Figure 11: Physical map of Genomic DNA around site of translocation breakpoint. Genomic DNA from normal and the translocation patient was digested with Sfi1, Ksp1, Eag1 and combinations of these enzymes, separated by PFGE, blotted, and probed with the PGK1 probe and 6E1 left end probe to generate this physical map. The normal Sfil and Kspl PFGE fragments are approximately 250 kb in size. PGK1 detects an approximately 700 kb altered fragment, and a ~900 kb altered Ksp1 fragment, representing the der(2) chromosome. The 6E1 left end probe identifies a ~350 kb altered Sfi1 fragment representing the der(X) chromosome. No additional altered Ksp1 band was detected with the 6E1 left end probe and the separation parameters tested and may be smaller or larger than the fragments resolved.

hybridization performed by Susan Allen in Barbara Trask's laboratory demonstrated that the lambda clone detected both derivative chromosomes and therefore confirmed this localization (data not shown).

1.1 kb breakpoint spanning fragment

A 1.1 kilobase genomic clone spans the site of the translocation breakpoint. A 8 kilobase subclone of λ3 detected both altered Sfi1 fragments in the DNA of the C.G. translocation patient suggesting it spanned the site of the breakpoint. The region was narrowed ultimately to a 1.1 kb Sst1/HindIII genomic fragment containing the site of the translocation breakpoint. The 1.1 kb clone detects both altered SfiI fragments on PFGE analysis (Figure 12). In addition, altered genomic fragments were detected by standard Southern analysis in genomic DNA from the translocation patient digested with HindIII and EcoRI (Figure 13). The sequence of this fragment spanning the site of the translocation breakpoint was determined and contained no significant open reading frames or homology to known sequence (Figure 14). The focus of the work shifted to the identification of cDNAs in the region and will discussed in the next section.

Identification of cDNAs

I will briefly discuss two additional cDNAs in addition to the Menkes cDNA described in the next chapter which were identified in the PGK1 YACs. The cDNAs Mc2 and Mc6 were identified by direct hybridization of the gel purified breakpoint spanning the YAC 19CH10. The YAC, 19CH10, was separated from the yeast chromosomes by preparative PFGE, gel purified, glass-milk purified(Bio101), labeled, prehybridized with placental human DNA to decrease hybridization due to repetitive sequences, and hybridized to

a human placental λGT10 cDNA library (protocol adapted from D.Marchuk, pers. comm.) Thirty-four potential weak positives were picked, cDNA inserts from 1° plugs were PCR amplified with λGT10 primers, the products run on an agarose gel, and hybridized with a 19CH10 probe prepared as above. The same blot was stripped and rehybridized with PGK1 cDNA probe and thereby identified nine potential positives which did not hybridize with PGK1 cDNAs. Secondaries were subsequently platted, hybridization screened, and PCR amplification screened as before. Tertiaries, as above identified individual hybridizing lambda clones. Subsequent analysis indicated that two represented cDNAs which map to 19CH1 (Figure 7). One, Mc6, which did not map in the region of interest was not analyzed further. Partial sequence analysis of Mc2 indicated that it encoded phosphoglycerate mutase beta (PGAM-B) (Sakoda et al., 1988). The corresponding genomic DNA in the 19CH10 YAC hybridizing to the Mc2 was PCR amplified, cloned, and analyzed by sequence analysis by Dr.Sam Yang in Dr.Packman's laboratory and was found to represent a pseudogene of the PGAM-B gene. It was concluded based on their location and sequence analysis that Mc2 and Mc6 were not candidate genes for Menkes disease.

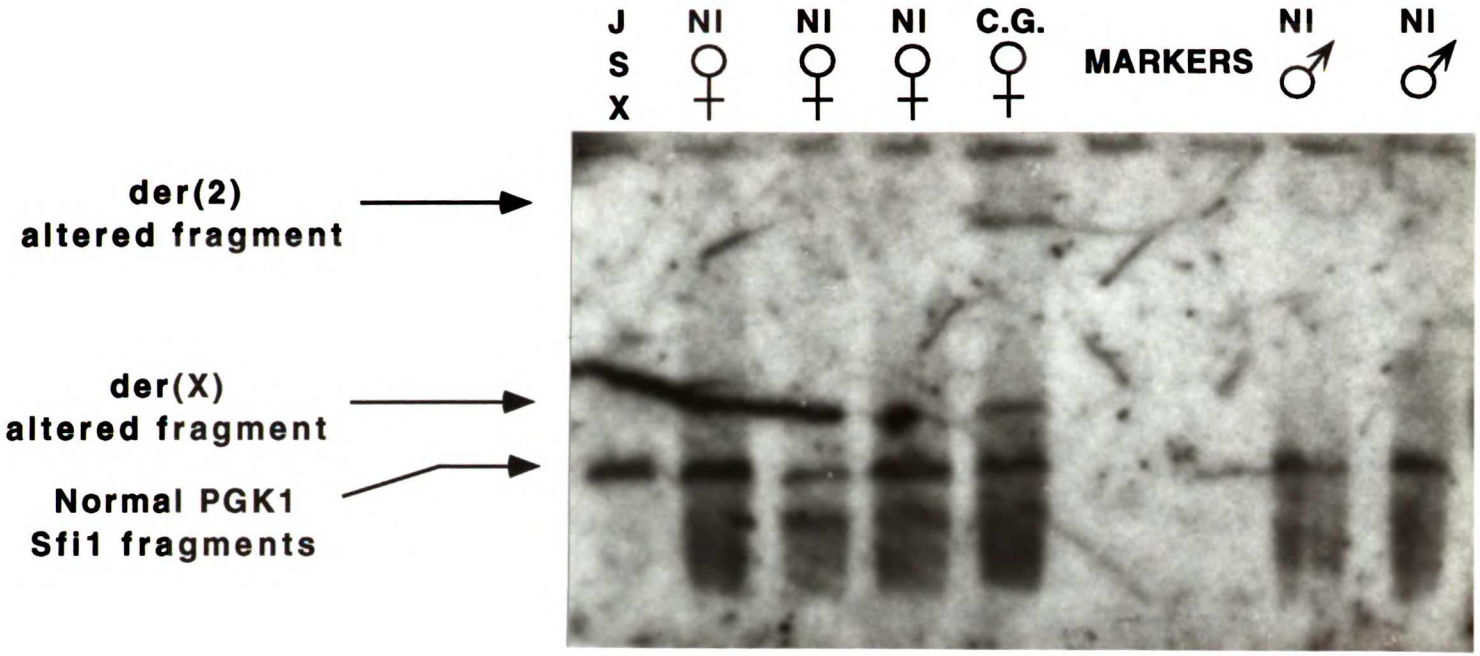
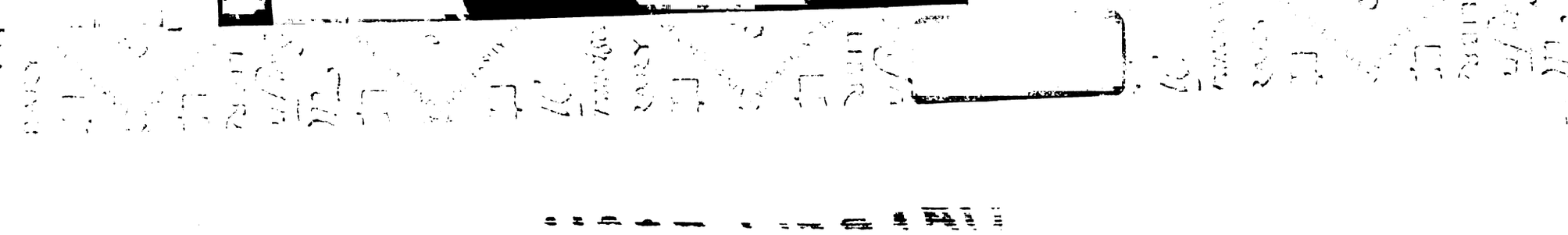


Figure 12: 1.1 kb genomic clone detects both altered PFGE Sfi1 fragments. A 1.1 kb genomic fragment (3.7 Sst1/HindIII 1.1 kb) was hybridized to the identical blot as in figures 4 and 9. Both the blot and the probe were pre-hybridized with placental human DNA. The genomic probe identifies the same normal PFGE fragments as PGK1 and the 6E1 left end probe. The probe also detects both the 700 kb altered PFGE fragment representing the junction fragment of the der(2) chromosome and the 350 kb altered PFGE fragment representing the junction fragment of the der(X) chromosome. The 1.1 kb genomic clone, therefore likely spans the site of the translocation breakpoint on the normal X chromosome.



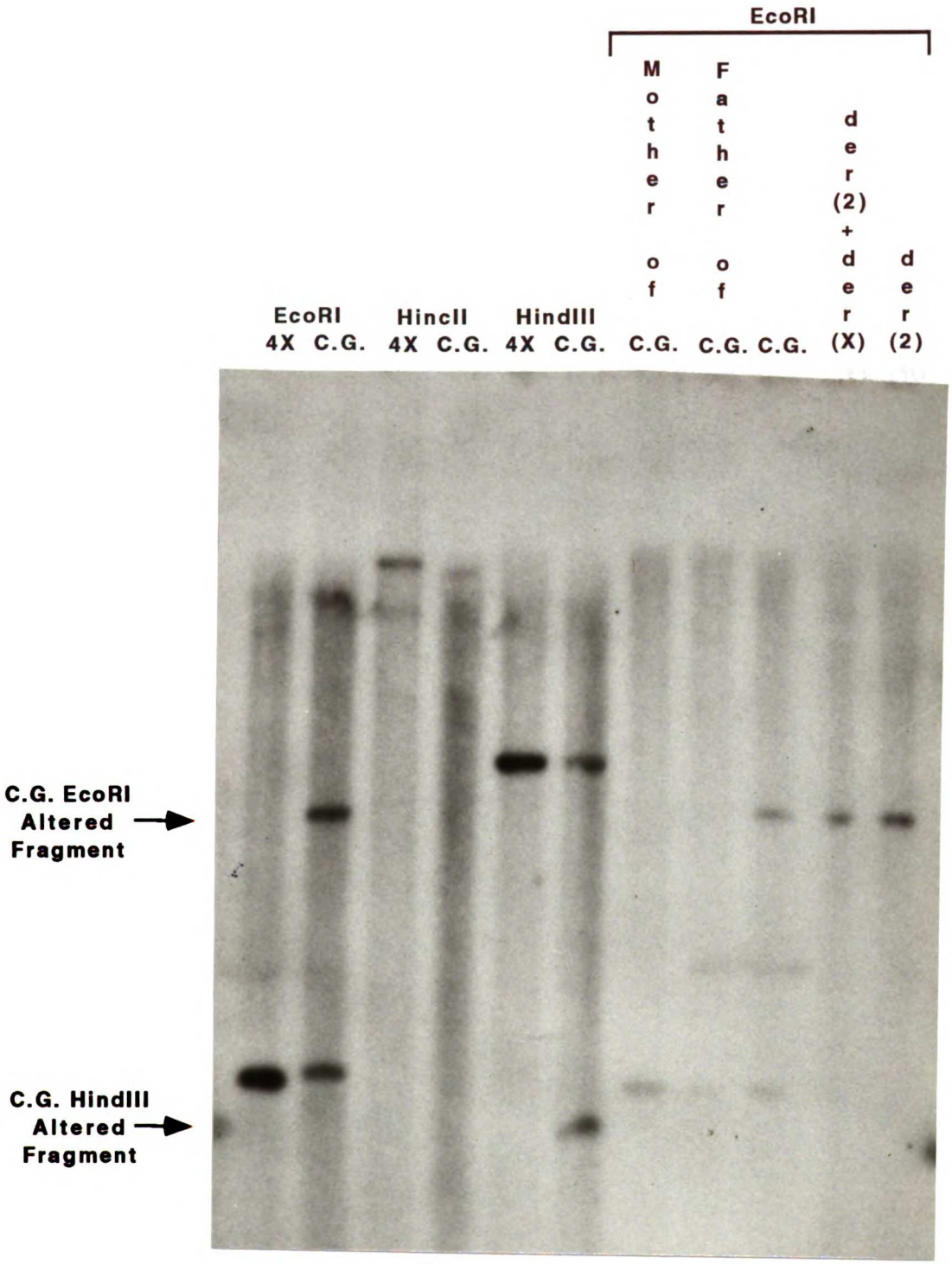

EcoRI
Mother of C.G.
Father of C.G.
der (2) + der (X)
der (2)
EcoRI 4X C.G.
HincII 4X C.G.
HindIII 4X C.G.
C.G. EcoRI Altered Fragment
C.G. HindIII Altered Fragment

Figure 13: Altered EcoR1 and HindIII restriction fragments in genomic DNA of Menkes patient, C.G., with the translocation. Genomic DNA was prepared from a control female cell line, 4X (GM012012B), containing four normal copies of the X chromosome, cell lines from the patient C.G. and her parents, as well as, somatic cell hybrids containing either the der(2) and the der(X) chromosome or the der(2) chromosome alone separated from the normal X chromosome in a hamster background. Ten micrograms of DNA digested with either EcoR1, HindIII, or HincII was run at 50V for 24 hrs in a 1% TAE gel and alkali blotted. The blot was probed with a non-repetitive subclone(3.7 HindIII/HincII top) of the 1.1 kb genomic fragment which crosses the site of the translocation breakpoint. Normal genomic fragments with the EcoR1 and HindIII of ~1.8 kb and ~5kb respectively were detected in 4X and the parents of C.G. (HindIII not shown for parents) An altered EcoR1 genomic fragment of ~4kb was noted in the DNA of C.G. and the somatic cell hybrids but not the normal controls and likely represents a junction fragment from the der(2) chromosome. Similarly, an altered HindIII fragment of ~1.6 kb is present in the DNA of C.G. HincII digestion was either incomplete, or the genomic fragment is outside the range of separation.

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   1  GAGCTCAAGCTATCTGCCCAACCACCTCAGCATCCCAAAGTGCTAGGATT   50
  51  ACAGGCGTGTGCCACCTGGCCTGAGGAAGTGATTTTTGGTTTTAAAATGA  100
 101  GAACAAAATCTGATTGGSTATGTTGGCAAATTGACATATTTGGGGGGGGT  150
 151  GGGTGCGGTGGAACCAGGTCTTGTTTAAGATGTACCGGATACAACGTATT  200
 201  TAAAGGGCAAGAATCCTTTACGTTGGTACTTTATCAGAAAAAGTATAGTA  250
 251  GTACCTAGAGCAATACCACCCAAGAATGGTCTGTGATGATGGAAATACTG  300
 301  TGTATCCGCCCTGTCTGGTAGGACTAACCACATTGGCCAATGAACACTTG  350
 351  AAATGTGGCTAGTGCAACCGAGGAACTGAATTTTTAATCTTATTTAATAG  400
 401  TTTTTTTTTTGTTTTAAAAAATAATCATCTAGTAAAATAGACTTTTTTTG  450
 451  GGTATATAGTTCTATGAAATTTNNCNNTAGCTTTGTGAATGTTAACACAT  500
 501  GTATNGATTTGGGTTACACCAACACAACCAGGACATAGCGCAGSCCTGTT  550
 551  ACCCCCTAAAAAACTCCCTCTTGCTACCATTTCCCTACCTCTAACCCCTG  600
 601  GAAACCACTGTTCTGTTCTCCATCTTCCCCTTTAGTTGGAAAGTTTAAAT  650
 651  TATTTACAGTCATTTTCTTAACCCTAAAATCACCTCAGGGGAGGGAAATT  700
 701  AAAGTTTAAAAGAGGCCTTCTTTAAAAACACAAGGACACAAAAGCCTTCA  750
 751  TTTGATGGTTGGCTACTCTTATATTGCATGTCTTTCAATACTATATGTCT  800
 801  CAACTATACGTGGTTTGACTATTCTGCATTTTTTTTTCATGGAAAATGTT  850
 851  ATATAGTATTATCCAAAACCAAATATAATTTATTTCCCCACTTCTGTTTT  900
 901  GGATCATTTATGCTAGTGATTTCCTCCATCCCTAAGAACCAGCTAGCCAA  950
 951  CTAGAATTATCAGAGTCTTAGAAACTTTCCATGCCATGGGTACATTTCAC 1000
1001  ATTTTCACCCAAATACACAGTCATCAATTATCAAGCTAAAGGAAGAAATA 1050
1051  AAAATATGTTCATTAAAATAAGTGAAAGATTTAACTATTGTAGACAATTT 1100
1101  AAAGAGAGATTTTCTATGCTGAATTACTTT  1130
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Figure 14: Sequence of 1.1 kb genomic clone spanning the site of the translocation breakpoint on the X chromosome. A 1.1 kb Sst1/HindIII subclone of λ3 which was determined to span the site of the translocation breakpoint was sequenced utilizing a Pharmacia sequenase™ kit. Both strands were sequenced for much of the clone although there a few small gaps where the sequence derives from one strand and there are ambiguities. The sequence contains one Alu repeat but has no other significant homologies to known sequences. Coding region identification computer programs, grail and gene-id, did not predict any exons within the sequence.

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Chapter 3

Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase

Isolation of a candidate gene for Menkes disease and evidence that it encodes a copper-transporting ATPase

Christopher Vulpe[1], Barbara Levinson[2], Susan Whitney[3], Seymour Packman[3] & Jane Gitschier[2]

Menkes disease is an X-linked disorder of copper transport characterized by progressive neurological degeneration and death in early childhood. We have isolated a candidate gene (Mc1) for Menkes disease and find qualitative or quantitative abnormalities in the mRNA in sixteen of twenty-one Menkes patients. Four patients lacking Mc1RNA showed rearrangements of the Menkes gene. The gene codes for a 1,500 amino acid protein, predicted to be a P-type cation-transporting ATPase. The gene product is most similar to a bacterial copper-transporting ATPase and additionally contains six putative metal-binding motifs at the N-terminus. The gene is transcribed in all cell types tested except liver, consistent with the expression of the Menkes defect.

The unique properties of the essential trace element copper require a delicate cellular balance between necessity and toxicity. Over thirty known proteins, as fundamental as the cytochrome oxidases and as specialized as dopamine β-hydroxylase, use the oxidative capacity of copper in their biological roles. But that same oxidative potential can cause extensive cellular damage through free radical production and direct oxidation of lipids, proteins and nucleic acids.

Two human disorders of copper transport reflect the conflicting requirements of copper homeostasis. In X-linked Menkes disease, the reduced activity of numerous copper-containing proteins may be responsible for the diverse clinical findings of progressive neurologic degeneration, poor temperature regulation, connective tissue defects, pallor, distinctive steely or kinky hair and death in early childhood[1]. At the cellular level, defective copper export results in the characteristic phenotype of copper accumulation in most tested cultured cells[1-3], except hepatocytes[4] and cultured chorionic villus cells[5]. In patients, copper deficiency results from the trapping of copper in intestinal mucosa, kidney and connective tissue, accompanied by failure of its distribution to other tissues[1].

In contrast, the clinical presentation of the autosomal recessive Wilson disease results from copper toxicity. Decreased copper export from the liver results in copper-induced chronic liver disease and contributes to pathologic changes in other tissues, especially the brain, kidney and eye[1]. Thus, defects in copper export underlie both the copper deficiency of Menkes disease and the toxicity of copper excess in Wilson disease. The underlying molecular defects, however, have remained unknown.

We now describe the identification of a candidate gene for the Menkes disease locus. Our approach centres on a female Menkes patient with a balanced X:2 translocation [46X,t(Xq13;2q32.2)], hypothesized to disrupt the Menkes locus at Xq13 (ref. 6). Linkage studies on Menkes disease families[7] and an additional chromosomal rearrangement described in a Menkes patient[8] support an Xq13 location for Menkes disease. Furthermore the murine homologue of Menkes, *mottled*, maps to a region of the mouse X chromosome bearing syntenic homology to the Xq13 region of the human X chromosome[9-10]. To identify the Menkes locus, we first determined the site of the translocation breakpoint on the X chromosome and then sought candidate genes in the region. We report the isolation of a gene encoding a putative copper-transporting ATPase, a finding which is physiologically consistent with the biochemical defect of Menkes disease.

Mapping the translocation region

Figure 1 depicts the location of the translocation breakpoint on the normal X chromosome and summarizes the strategy used to identify the Menkes candidate gene. We constructed a physical map of the genomic region around the site of the translocation breakpoint on the X chromosome. *DXS441* and *PGK1* were determined to be the closest available probes flanking the site of the translocation breakpoint, consistent with the finding of Verga *et al.*[11]. Alterations in the physical map of genomic DNA from the translocation patient suggested the translocation lay within 300 kilobases (kb) of *PGK1*. Therefore *PGK1*-containing yeast artificial chromosomes (YACs) were obtained from several sources and two (19CH10 and 6E1) were determined to span the site of the translocation breakpoint. Vectorette-[12] and *Alu*-

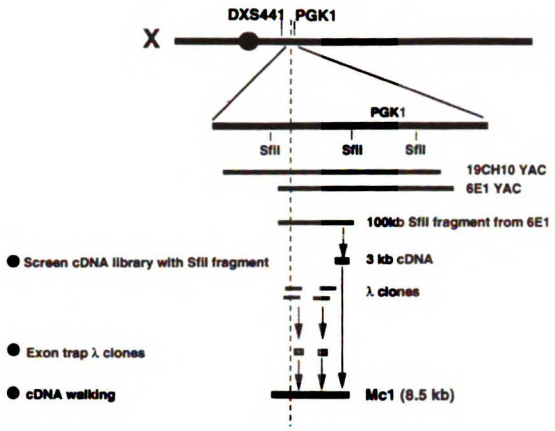

Fig. 1 Strategy for isolation of cDNAs near the translocation breakpoint. At the top is represented the normal human X chromosome, and the dashed line represents the position of the translocation breakpoint in the female Menkes patient. The position of two YAC clones (19CH10 and 6E1), several λ clones, and the 100 kb *Sfi*I fragment are also shown. The relative locations of the exon-trapped clones and the initial 3 kb cDNA clone are indicated. The sizes of the λ and cDNA clones are not drawn to scale. The extent of the primary Mc1 transcript, which is processed to an 8.5 kb mRNA, is also indicated.

polymerase chain reaction (PCR)[13] probes were used to identify genomic λ clones and a 1.1-kb subclone spanning the site of the translocation breakpoint. Consistent with the results of Tumer *et al.*[14], we located the translocation breakpoint ~25 kb from the proximal *Sfi*I site of a ~125-kb *Sfi*I fragment. A detailed presentation of the physical map and the sequence of the region surrounding the breakpoint will be described elsewhere (Vulpe *et al.*, in preparation).

Isolation of cDNA clones

Two independent methods of gene identification, direct YAC screening of cDNA libraries and exon trapping, converged to identify one locus in close proximity to the translocation breakpoint. In one approach, a placental cDNA library was screened directly with a 100 kb fragment of YAC 6E1 extending from the end of the YAC to the distal *Sfi*I site (Fig. 1). This fragment contains the site of the translocation breakpoint but not the highly expressed *PGK1* locus. It identified a 3 kb cDNA which mapped ~80 kb from the translocation breakpoint. Sequence analysis of the cDNA clone and of the corresponding genomic DNA suggested the entire clone represented the 3'-untranslated region of a gene, including the polyA+ tail.

In the second approach, we utilized exon trapping vectors[15] to identify coding regions in two sets of genomic λ clones (Fig. 1), one set encompassing the breakpoint and one set 20 kb distal. One exon-trapped clone with an open-reading frame was obtained from each set of λ clones.

Each of the three cDNAs detected an 8.5 kb transcript by northern blot analysis and were used to isolate a total of eleven different overlapping cDNAs from an unamplified fibroblast library (kindly provided by Jay Ellison and Larry Shapiro). Together these comprise the full-length cDNA designated Mc1, for Menkes cDNA 1.

We estimate that the Mc1 gene spans roughly 100 kb of genomic DNA and is likely to cross the translocation breakpoint. An exon beginning at position 267 of our sequence starts approximately 2 kb distal to the site of the translocation breakpoint, as determined by PCR analysis of genomic DNA. Also, 5'-most cDNA probes do not hybridize to the most proximal portions of the λ clones isolated, suggesting the 5'-most exon or exons lie at least 10 kb proximal to the translocation breakpoint.

Expression of the Mc1 gene

Hybridization of the Mc1 cDNA to a northern blot containing polyA+ RNA showed expression of the Mc1 gene in heart, brain, lung, muscle, kidney, pancreas, and to a lesser extent placenta (Fig. 2). Little or no Mc1 mRNA was detected in liver. Additional studies show expression in fibroblasts and lymphoblasts (data not shown). A preliminary investigation also suggests that a gene homologous to Mc1 is found in many other mammalian species and in yeast (data not shown).

Altered expression of *Mc1* in Menkes patients

Abnormal expression of the Mc1 gene in fibroblast cell lines derived from the female Menkes translocation patient and other severely affected Menkes patients is shown in Fig. 3. Fibroblasts express the cellular phenotype of aberrant copper transport and therefore are an appropriate cell type for this analysis[1]. Lane C shows the expression level of Mc1 mRNA in a control fibroblast line. Fibroblasts from the female Menkes translocation patient (lane 1) show no detectable expression of this transcript. Five of six additional fibroblast lines from unrelated Menkes patients show alterations in the expression of this gene. The sample in lane 4 shows no detectable Mc1 mRNA, while sample 7 shows a greatly decreased amount. The Mc1 transcript in sample 3 is of a smaller size than expected. Samples 2 and 5 produce greatly reduced levels

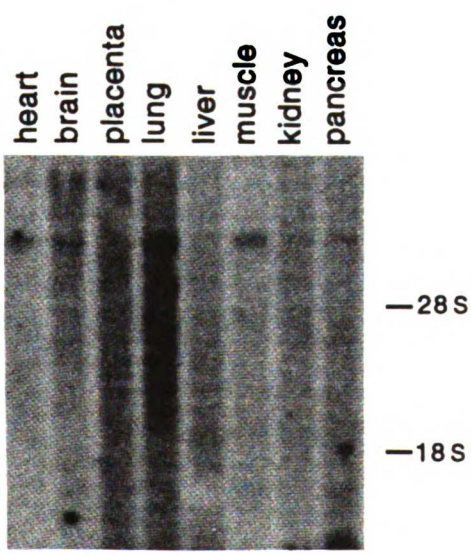

Fig. 2 Expression of Mc1 in human tissue. A northern blot, containing 2 μg polyA+ RNA from multiple human tissues, was hybridized to a partial Mc1 cDNA probe. Mc1 detects a widely expressed, 8.5 kb transcript, which is noticeably absent from liver.

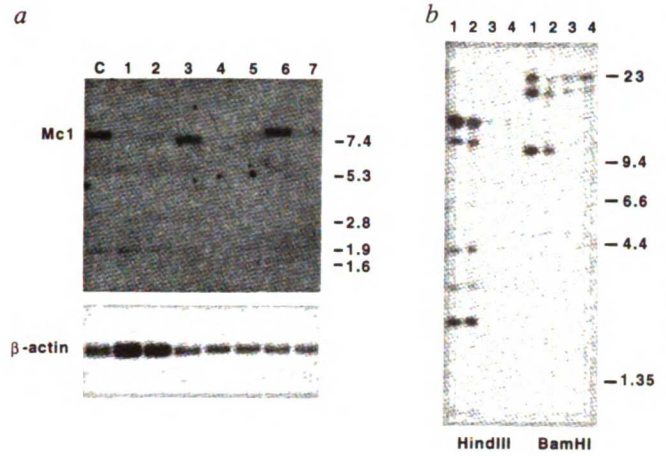

Fig. 3 Evidence for abnormalities in expression and structure of the Mc1 gene in Menkes patients. *a*, A northern blot containing 2 μg polyA+ fibroblast RNA was hybridized to a partial Mc1 cDNA probe and β-actin cDNA as an internal control probe. The RNA samples on the blot are the following fibroblast cultures: C, normal human (WI-26), SV40 transformed; 1, female Menkes translocation patient, SV40 transformed; 2, Menkes patient (GM1981), SV40 transformed; 3, Menkes patient (GM220), non-transformed; 4–7, non-transformed fibroblasts from unrelated Menkes patients. The lower molecular weight, faintly hybridizing bands, may represent non-specific hybridization to ribosomal RNA. *b*, A Southern blot containing 10 μg genomic DNA, digested with *Hin*dIII and *Bam*HI and run on 0.8% agarose gel, was hybridized to a probe from the Mc1 cDNA that includes nucleotides 149 to 2941 under standard conditions. Samples included DNA from two unaffected males (lane 1 and 2) and two brothers affected with Menkes disease (lanes 3 and 4). The asterisk indicates the altered band in the two affected boys in the *Hin*dIII digest.

of two hybridizing mRNAs, one of normal size and another of larger size. Only the Mc1 transcript in lane 6 appears to be of normal size and abundance. Hybridization with a β-actin probe controlled for the presence of mRNA in each lane. Further studies revealed significant qualitative and quantitative abnormalities in 10 of 14 additional patients examined (data not shown).

Genomic rearrangements of the Mc1 gene

Genomic DNA from 26 patients was prepared and analysed for gene rearrangements by Southern blot analysis. Aberrations were noted for four unrelated patients. Figure 3*b* demonstrates missing or altered bands in DNA from one patient and his affected brother with two different restriction enzymes. An altered band, indicated by the asterisk, is observed only with the 5'-most cDNA probe, whereas the deleted bands are noted with probes from the central portion of Mc1. A probe from the 3' untranslated region showed no abnormalities in these two patients (data not shown). Together the results indicate that there is an intestitial deletion of the Mc1 gene in the pair of brothers. In all four of the unrelated patients with gene rearrangements, no Mc1 mRNA was detectable by northern blot (data not shown).

Sequence analysis

Both strands of the Mc1 composite cDNA were sequenced in at least one cDNA and in multiple cDNAs for much of the gene. We identified one open reading frame of 4,500 bp encoding a predicted protein of 1,500 amino acids (Fig. 4). The sequence 5' of the proposed initiating methionine codon has stop codons in all three reading frames.

Fig. 4 Sequence of the Mc1 cDNA and derived amino acid sequence. The six heavy-metal binding motifs are indicated by the shaded areas, and the six putative transmembrane spanning domains, as determined by a hydrophobicity plot[52], are indicated by boxes. The phosphatase domain lies between transmembrane domains 2 and 3. Transmembrane domain 4 includes the residues CPC, part of the proposed transduction domain. The phosphorylation motif DKTGTIT is located at position 1044, and the phosphorylated aspartic acid is indicated in bold. Sequences involved in binding ATP are located between the phosphorylation domain and the fifth transmembrane domain.

Continued▶

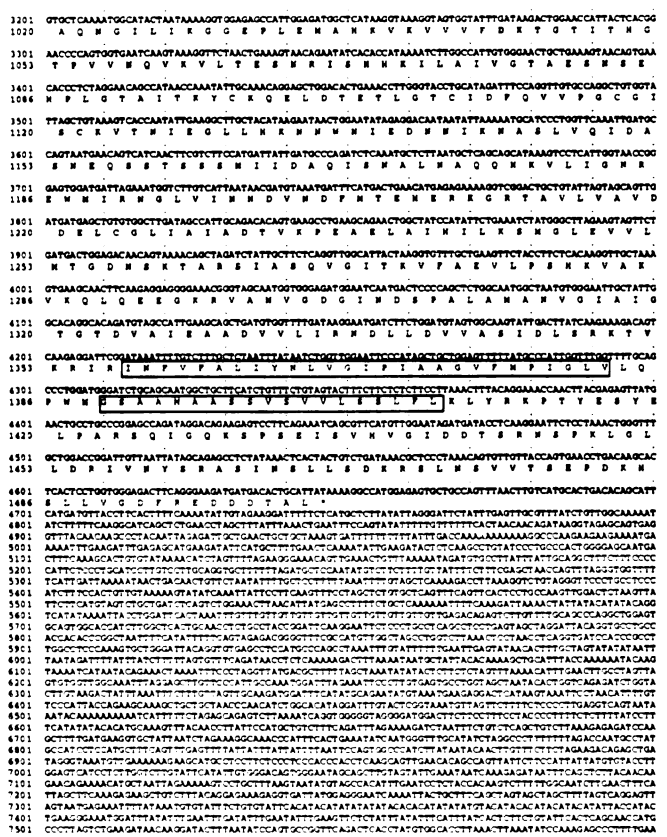

Fig. 4 Continued from previous page.

We note ten tandem copies of the trinucleotide repeat GTT in the 3.8 kb 3' untranslated region. The recent implication of trinucleotide repeats in several diseases[16] led us to investigate whether the repeats were amplified in Menkes patient DNA. PCR amplification of genomic DNA in sixteen patients showed no substantial alterations in copy number.

Predicted amino acid sequence of Mc1

Comparison of the predicted Mc1 protein sequence to other sequences in the databases indicates that the Mc1 gene encodes a cation-transporting ATPase of the P-type. P-type ATPases form a family of integral membrane proteins which utilize an aspartyl phosphate intermediate to transport cations across a membrane. The predicted Mc1 protein shows the greatest similarity to the *Enterococcus hirae* Cu^{++}-exporting ATPase[17], formerly reported as the K+ ATPase of *Streptococcus faecalis*[18] (Fig. 5). It is also closely related to a subfamily of prokaryotic ATPases including cadmium transporters[19,20], potassium transporter[21], and the nitI gene product, a predicted ATPase of unknown cation specificity from *Rhizobium meliloti*[22].

The Mc1 derived amino acid sequence has all of the features described in other members of the P-type ATPase family. These include the following: (i) a cation channel, formed by transmembrane hairpin loops; (ii) a stalk, formed by extensions of the transmembrane α-helices into the cytoplasm; (iii) an ATP binding domain; (iv) a phosphorylation domain which contains a conserved, phosphorylated aspartic acid; (v) an adjacent transduction domain; and (vi) a phosphatase domain, which removes the phosphate from the aspartic acid as part of the reaction cycle[23–27] (Figs 4, 6).

One structural feature that Mc1 shares with its subfamily is the presence of only six transmembrane domains, as predicted by hydrophobicity analysis (see Fig. 4). All other P-type ATPases are postulated to contain additional transmembrane domains near the C terminus[28].

Sequence alignment of the putative Mc1 protein with the bacterial cadmium, copper and potassium transporters, as well as with representative members of the other families, further illustrates the remarkable similarity between the Mc1 putative protein and members of the ATPase family. Figure 7*a* shows alignments for selected conserved regions of proposed functional significance, including portions of the phosphatase domain, the phosphorylation domain, and the ATP-binding domain.

The phosphatase domain, located between the second and third transmembrane domains, typically contains a four residue motif, TGES, which may have a role in removing the phosphate from the phosphorylated aspartic acid (Fig. 7). This site also binds vanadate, an analogue of phosphate and an inhibitor of P-type ATPases[29]. The Mc1 product has an alanine as the last residue of this motif instead of the canonical serine. Substitution of alanine for serine in the proton-transporting ATPase of yeast resulted in a 17% decrease in proton transport[30].

In the phosphorylation domain, Mc1 contains the invariant aspartic acid proposed to be phosphorylated in the cation transport cycle and a conservative change of isoleucine for leucine in the conserved DKTGTLT sequence. Most eukaryotic transporters have cysteine and serine (or threonine) residues immediately preceding the aspartic acid residue; however Mc1, like most prokaryotic transporters, does not have these residues.

An invariant proline residue is situated 43 amino acids N-terminal of the phosphorylated aspartic acid residue. This proline may be involved in the transduction of the energy of ATP hydrolysis to cation transport[31]. The residue following the proline is critical for ion binding in the Ca^{++}-transporting ATPase of sarcoplasmic reticulum[32]. In most cation transporters, a hydrophobic residue such as valine or isoleucine immediately precedes the proline, while a glutamic acid or a valine follows the proline. Strikingly, cysteine residues immediately surround the proline in the Mc1, cadmium, copper and NitI ATPases. These proteins have an additional proline eight residues toward the C-terminus. In contrast, the *E.coli* potassium transporter does not have the flanking cysteines nor the additional proline, indicating that these are not general features of prokaryotic P-type ATPases. Thus, the presence of these residues may suggest a specificity for metal ions.

Figure 7*a* also shows a multiple sequence alignment of one of the most conserved dispersed sites responsible for ATP binding in P-type ATPases[25,29]. The Mc1 sequence has remarkable similarity to this region with several conservative changes. The sequence of this particular loop is homologous to the ATP-binding site of phosphofructokinase.

The amino termini of P-type ATPases is one of the most divergent domains and may be involved in initial ion binding. The amino-termini of the Mc1 gene product and the cadmium transporters contain 23 amino-acid motifs with a pair of conserved cysteine residues. As Fig. 7*b* demonstrates, this motif has been noted in other proteins that bind or transport metals, as well as in the NitI gene product, and may constitute a metal binding site[19,33]. Mc1 contains six closely related repeats of this motif spaced throughout the 600 amino acid N-terminus (Fig. 4), in contrast to the presence of a single motif in other published sequences.

Discussion

Several lines of evidence suggest that Mc1 is the gene responsible for Menkes disease: (i) the Mc1 gene maps to Xq13, and is likely to cross the translocation breakpoint in a female Menkes patient. (ii) No Mc1 transcript is detectable in the translocation patient, and qualitative or quantitative changes in Mc1 mRNA were observed in fifteen of twenty additional unrelated Menkes patients. iii) Genomic DNA rearrangements, including one interstitial deletion of the Mc1 gene, were found in 4 of 22 unrelated Menkes patients. (iv) The Mc1 mRNA is expressed in most tissues, but is notably absent in the liver, a tissue which does not show the Menkes copper sequestration phenotype. (v) The derived amino acid sequence predicts a protein with metal-transporting ATPase activity. The remarkable similarity of Mc1 to a bacterial copper efflux protein and the cellular phenotype of Menkes disease further suggest that the Mc1 protein transports copper.

Several features of the Mc1 protein may play a part in copper specificity. The N terminus of Mc1 contains six putative metal binding motifs. We suggest these motifs bind copper because they appear to be more closely related to each other than to the cadmium and mercury binding motifs (Fig. 7). They may serve as redundant, transient copper binding domains, or alternatively the six copies may constitute a gauge to modulate copper transport as a function of intracellular copper concentration[34]. The sequence similarity of the remaining 900 amino acids of Mc1 to an *E. hirae* copper efflux P-type ATPase further supports a role for Mc1 in copper transport. This conservation includes a potential copper binding site of two cysteines flanking a proline in the predicted ion channel.

A defect in the proposed Mc1 copper-transporting ATPase could explain the biochemical defect in cultured Menkes cells. Menkes cells have a specific defect in copper efflux, with transport of cadmium and zinc unaffected in mutant cultured cells[35,36]. Detailed copper efflux and cell fractionation studies suggest that the copper accumulation of Menkes cells results from a subcelluar rather than plasma membrane defect in copper export[36–38]. Thus we propose that the copper-transporting ATPase is inserted in the membrane of a subcellular compartment.

The identification of a copper-transporting ATPase may have implications for other inherited disorders in copper metabolism. In the human disease X-linked cutis laxa, patients have connective tissue and copper transport defects similar to Menkes patients, but do not have severe neurologic abnormalities[39]. This disorder may represent an allele of Menkes resulting in a milder form of the disease.

A murine homologue, *mottled*, of Menkes disease has been described[1]. *Mottled* cells in culture show cellular defects of copper export resulting in copper accumulation indistinguishable from Menkes cells[1,36,40,41]. Alleles of the mottled locus show considerable phenotypic variability including the mainly hair pigmentation defects of Mo^{pewter}, primarily connective tissue defects of $Mo^{blotchy}$, the neurological defects of $Mo^{brindled}$, and the prenatal lethality of $Mo^{tortoiseshell}$. If the Mc1 gene is responsible for Menkes disease, the relationship between *mottled*, X-linked cutis laxa and Menkes disease can be investigated at the molecular level. These studies may provide further insights into copper metabolism.

Wilson disease may represent defects in a gene related to the Menkes disease locus. Menkes and Wilson disease contrast not only in their mechanisms of pathogenesis, copper deficiency versus toxicity, but also in the site of expression of the defect. Wilson disease primarily affects the liver while in Menkes disease the liver remains largely unaffected and does not accumulate copper[1]. Different mechanisms of copper transport in the liver have been suggested to explain the antithetical relationship between

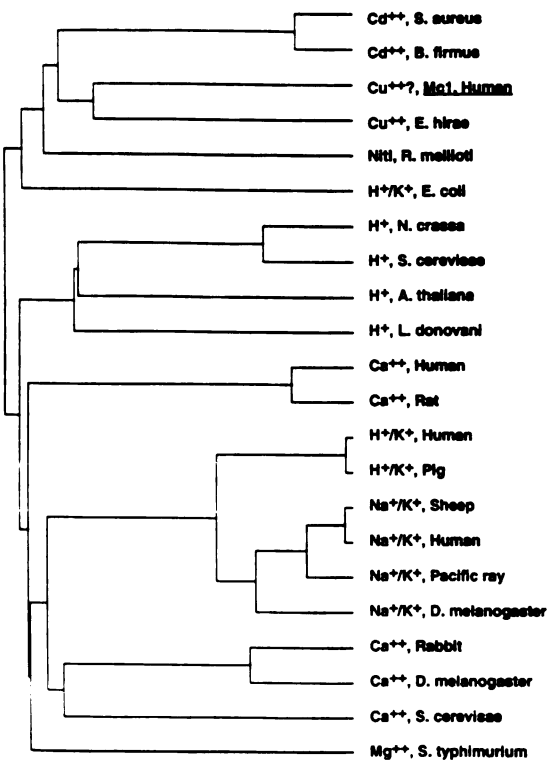

Fig. 5 Graphic representation of sequence relationships between cation-transporting ATPases. The tree is the output of the program Pileup from the Genetics Computer Group[53]. The sequences in the tree are arranged according to amino acid similarity, and the length of each branch is a measure of how closely related two sequences are. As seen in this tree, ATPases generally form sub-families according to the particular ion transported. From the top to bottom of the figure, the Accession numbers for these sequences (obtained through GenBank, EMBL, SwissProt or PIR) are the following: A32561, M90750, L06133 (this paper), J02729, M24144, Pwecbk, A25939, A25823, P20649, J04004, A30802, B28065, A36558, A31671, Pwshna, A24414, S00503, S03632, Pwrbfc, A36691, Pwbyr1, B39083.

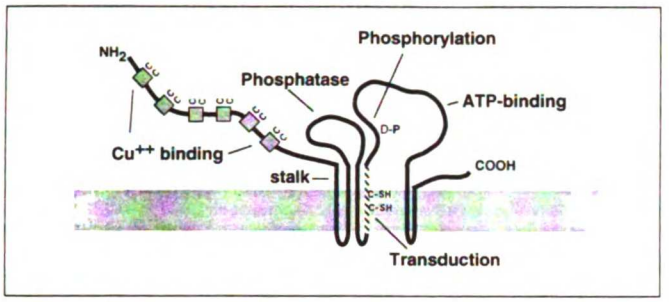

Fig. 6 Model for the proposed gene product of the Mc1 gene. The boxes with paired cysteines (C) represent the putative copper-binding motifs. This proposed model is based on models for the Ca^{++}- and Cd^{++}-transporting ATPases[24,33] and the yeast proton pumping ATPases[29].

Menkes and Wilson disease. Indeed, copper export in cultured hepatocytes and fibroblasts differ in that both show export suggestive of at least a two component system but fibroblasts show a greater rate of efflux than isolated hepatocytes[34]. Our results indicating undetectable expression of the Mc1 gene in liver support the hypothesis of alternative transport mechanisms functioning in the liver. Possibly Wilson disease results from defects in a related copper transporter which functions in the liver.

If the Menkes candidate gene product represents a copper transporter, it bridges a gap in our knowledge of eukaryotic copper homeostasis. Both prokaryotic and eukaryotic cells appear to maintain copper balance by sequestration of extra copper in an inert form bound to specialized proteins or by exporting it from the cell. In prokaryotes two systems illustrate these themes: in *Pseudomonas syringae* excess copper remains bound to proteins (copA-D) in the periplasmic space[42], while in *E.coli* excess copper is exported through a combination of plasmid borne (*pco*) and chromosomal (*cut*) genes[43,44]. In eukaryotes, the metallothioneins, small cysteine-rich proteins, sequester copper[45]. Copper transporters have not been identified previously in eukaryotes; consequently the Mc1 gene product may represent the first eukaryotic copper transporter. As such, it suggests approaches to identify other transporters and provides a basis for future studies of eukaryotic copper transport.

We note that two other laboratories have identified cDNA clones corresponding to the 5' region of Mc1[46,47]. They also report evidence for genomic rearrangements[46] and altered mRNA expression in Menkes patients using these cDNAs[47]. These findings provide corroborating evidence that the Mc1 gene is the Menkes (MNK) gene.

a ALIGNMENT WITH P-TYPE ATPASES

	PHOSPHATASE	TRANSDUCTION//PHOSPHORILATION	ATP BINDING
Cd++ S. aureus	GEKIAMDGITVNGLSA...VNQAAITGESVPV	CPCALVISTP//VAFDKTGTLT	IGDIVNDAPALAA
Cd++ B. firmus	GQKIAMDGVVVSGYSA...VNQTAITGESVPV	CPCALVISTP//IAFDKTGTLT	VGDGVNDAPALAA
Cu++?, Mc1, Human	GQKFPVDGRVIEGNSM...VDESLITGEAMPV	CPCSLGLATP//VVFDKTGTIT	VGDGINDSPALAN
Cu++/K+, E. hirae	GDKMPTDGTIDKGNTI...VDESAVTGESKGV	CPNALGLAIP//IMLDKTGTLT	VGDGINDAPSLAR
Ntl I. R. mellioti	GERVPVDGAVLSGTSD...LDRSVVNGESRPT	CPCALGLAVP//VLLDKTGTLT	VGDGINDAPVLAR
H+/K+, E. coli	GDIIPCDGEVIEGGAS...VDESAITGESAPV	IPTTIGGLLS//LLLDKTGTIT	TGDGTNDAPALAQ
H+, S. cerevisae	GTVIPTDGRIVT.EDCFLQIDQSAITGESLAV	VPVGLPAVVT//LCSDKTGTLT	TGDGVNDAPSLKK
Na+/K+, Human	GDRIPADLRIISANGC..KVDNSSLTGESEPQ	VPEGLLATVT//ICSDKTGTLT	TGDGVNDSPALKK
H+/K+, Human	GDRVPADIPILAAQGC..KVDNSSLTGESEPQ	VPEGLLATVT//ICSDKTGTLT	TGDGVNDSPALKK
Ca++, Rabbit	GDKVPADIRILSIKSTTLRVDQSILTGESVSV	IPEGLPAVIT//ICSDKTGTLT	TGDGVNDAPALKK
Mg++, S. typhimurium	GDLVPADVRLLASRD..LFISQSILSGESLPV	TPEMLPMIVS//LCTDKTGTLT	LGDGINDAPALRD

b ALIGNMENT WITH METAL BINDING MOTIFS

Cd++ B. firmus	VQGFTCANCAGKFEKNVKQLSGV
Cd++ S. aureus	VQGFTCANCAQKFEKNVKKIPGV
Hg++, Nrl merTB	VPGMTCAACPITVKKALSKVEGV
Hg++, Du1358 merP	VPGMTCSACPITVKKAISKVEGV
Hg++, Tn501 merA	ITGMTCDSCAAHVKEALEKVPGV
Hg++, T fer merC2	ITGMTCAHCAHSVEKALLGING1
Cu++?, Mc1-hmb1	VEGMTCNSCVWTIEQQIGKVNGV
Cu++?, Mc1-hmb2	VEGMTCNSCTSTIEGKIGKLQGV
Cu++?, Mc1-hmb4	IDGMTCNSCVQSIEGVISKEPGV
Cu++?, Mc1-hmb5	VTGMTCASCVANIERNLRREEGI
Cu++?, Mc1-hmb6	VRGMTCASCVHKIESSLTKHRGI
Cu++?, Mc1-hmb3	IDGMGCKSCVENIESTLSALQYV
Ntl I. R. mellioti	VPNAYCUTCIATIEGALRAKFEV

Fig. 7 Alignment of the Mc1 derived amino acid sequence to sequences of other proteins. *a*, The Mc1 sequence is aligned to all members of the prokaryotic subfamily and a representative member of other subfamilies, shown in Fig. 5. Residues 853–881 are included in the phosphatase domain. The transduction and phosphorylation domains include residues 1000–1050, where the // represents thirty intervening amino acids. Amino acids 1299–1311 are included in one of the ATP-binding domains. *b*, Six copies of a 23 amino acid motif from Mc1 sequence are aligned to similar sequences from selected proteins that bind metals. The sequences were aligned in order of their relatedness by the Pileup program[53]. Accession numbers for the sequences in *a* are given in the legend of Fig. 6, and numbers for *b* include A32561, M90750, K03089, M24940, K00031, X57326 and M24144.

Methodology

Cells and cell culture. Cultured skin fibroblasts from patients and unaffected individuals were propagated in Dulbecco's modified Eagles H21 medium, with 10% fetal calf serum as described[40]. Patient lines were derived from children with clinical features of Menkes disease following informed consent or from the Mutant Genetic Cell Repository (Camden, GM1981 GM220). All fibroblast cultures were shown to accumulate excessive concentrations of copper, by atomic absorption-spectrophotometry of whole cell extracts, as described[40]. A control SV40-transformed fibroblast line (WI-26) was obtained from the UCSF Cell Culture Facility. Fibroblasts from a female translocation patient[6] and GM1981 lines were transformed by pSV40ori by standard protocols[48].

RNA preparation and northern blot analysis. PolyA+ RNA was isolated from 1×10^7 cultured cells using a Pharmacia QuickPrep Micro RNA Purification Kit. Approximately 2 μg of RNA was run on a 1% agarose gel and blotted. A multiple tissue human Northern blot was purchased from Clonetech. Both blots were hybridized as described[49] with a partial Mc1 cDNA probe corresponding to bp 1150–2940.

Screening of a cDNA library with a 100 kb *Sfi*I fragment. A size-selected placental oligo(dT)-primed cDNA library in λgt10 was a gift from Jeff Edman. 1×10^6 recombinant plaques from the placental cDNA library were screened with the 100 kb *Sfi*I pulsed-field gel fragment as described[50]. 200 ng of the Geneclean (Bio 101) purified fragment was radiolabeled by random-priming using 250 μCi of both ^{32}P-dCTP and ^{32}P-dATP. The probe was mixed with sonicated human placental DNA (Sigma) at a final concentration of 1 mg ml^{-1}. The mixture was boiled for 10 min, quick cooled on ice and brought to 0.1 M NaCl. The probe was pre-annealed at 65 °C for 15 min and then added to the filters in hybridization buffer (0.5 M $NaPO_4$ pH 7, 7% SDS, 0.1 mg ml^{-1} sonicated salmon sperm DNA). The filters were hybridized at 65 °C for 36 h and washed in 2×SSC, 0.1% SDS at room temperature for 30 min, followed by a wash at 0.5×SSC, 0.1% SDS at 65 °C. Duplicate weakly hybridizing plaques were picked, eluted in SM buffer overnight, and 5% of the eluate amplified in PCR reactions using vector primers flanking the inserts. The PCR products were electrophoresed on 2% agarose, blotted on Hybond N$^+$ filters and probed with the radiolabelled 100 kb *Sfi*I fragment. Positive eluates were replated and the plaques rescreened until plaque purified. The single resulting positive cDNA was subcloned into pBluescript (Stratagene).

Exon trapping. Subclones of two genomic λ clones which span the site of the translocation breakpoint were individually cloned into pSPL1[15]. Subclones of three overlapping λ clones, located 20 kb distal to the breakpoint, were shotgun subcloned into pSPL1. For each pSPL1 construction, several hundred clones were picked to ensure that genomic fragments were cloned in both orientations. 20 μg of the pooled pSPL1 clones were Qiagen-prepared and electroporated at 800 V, 300 kf in a BRL cell-porator™ into COS7 cells representing one-half of a 70% confluent T75 flask. After 72 h of growth, the cells were harvested and RNA was prepared using Pharmacia QuickPrep™ RNA kit.

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First strand cDNA was made from one third of the RNA using Invitrogen cDNA cycle kit™ with the SA4 primer (5'GCA CCT GAG GAG TGA ATT GGT CG3'). Half of the first-strand cDNA was amplified using the SA4 and SD2 primers with suggested conditions with other primer sequences as described[25]. Potential amplification products larger than 375 bp vector product were isolated by Na45 paper (Sigma) and reamplified with the nested primers SA1 and SD1. Products of the nested amplification were gel purified and cloned into *Xcm*I-digested pDK101[26] and sequenced. We identified one 300 bp exon-trapped clone from the shotgun subclones and one 1.3 kb clone, comprising several exons, from an individually subcloned 8 kb *Sst*I fragment which contains the site of the translocation breakpoint.

cDNA walking. An unamplified oligo(dT)- and random-primed human fibroblast library in λgt10 was a gift from Jay Ellison and Larry Shapiro. This library was screened with a cloned insert from the *Sfi*I generated cDNA and the exon-trapped clones. It was also re-screened with resulting cDNAs to establish the composite Mc1 cDNA contig. The positive plaque eluates were amplified by PCR using λgt10 flanking primers and the products cloned into the pDK101[26] or pBluescript II (Stratagene) vectors.

DNA sequencing. The plasmid cloned Mc1 cDNAs were sequenced on both strands with modified T7 DNA polymerase (Sequenase 2, USB) using vector and Mc1 specific primers. Some of the clones were also sequenced using an ABI 373B automatic DNA Sequencer. The Mc1 sequence has been deposited in GenBank (accession number L06133).

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Chapter 4

The mottled gene is the mouse homologue of the Menkes disease gene

The mottled gene is the mouse homologue of the Menkes disease gene

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The mottled mouse has been proposed as an animal model for Menkes disease, an X-linked disorder of copper transport. The recent isolation of a copper-transporting ATPase gene responsible for Menkes disease has allowed us to test this hypothesis. Here we report the isolation and sequence of the mouse homologue of this gene. We show that two mottled *(Mo)* alleles, dappled *(Mo^{dp})* and blotchy *(Mo^{blo})*, have abnormalities in the murine mRNA and that *Mo^{dp}* has a partial gene deletion. These studies prove that the mottled mouse is the murine model for Menkes disease, providing the basis for future biochemical and therapeutic studies.

More than forty years ago, the light and dark coat colour variegation in female mutant mice led to the identification of the first allele of the X-linked mottled locus *(Mo)*[1]. The mottled alleles became a focus of studies on mammalian X-linked traits and in 1961, Mary Lyon proposed X-inactivation to explain the coat colour phenotype of mottled heterozygotes[2]. Coat colour changes are but one manifestation of mutations at the mottled locus. The alleles display considerable phenotypic variability in the hemizygous males, ranging from isolated coat colour changes in pewter (Mo^{pre}), connective tissue defects of blotchy (Mo^{blo}) and viable brindled (Mo^{vbr}), severe neurological disease and perinatal lethality of brindled (Mo^{br}) and macular (Mo^{ml}), to the prenatal lethality in dappled (Mo^{dp}) and tortoiseshell (Mo^{to})[3].

A flaw of copper metabolism underlies the characteristic defects of the mottled mice. In 1974, D. M. Hunt demonstrated decreased copper levels in the brain of Mo^{br} males and proposed that a primary copper deficiency leads to secondary deficiencies in copper enzymes and to the diverse phenotypes in the mottled mutants[4]. Cells cultured from many tissues, except the liver[5], exhibit a primary defect in copper export resulting in cytosolic copper accumulation[6,7]. Systemic copper deficiency results from the trapping of copper in some tissues, most notably intestine and kidney, leading to a failure of copper delivery to most other tissues[8]. Consequently, most organs have reduced activity of copper enzymes, such as dopamine β-hydroxylase[9,10], cytochrome oxidase[9,11], lysyl oxidase[12,13] and superoxide dismutase[9].

The mottled mouse has many phenotypic, biochemical and genetic similarities to X-linked Menkes disease of man. Menkes disease patients manifest similarly diverse clinical findings including hypopigmentation, connective tissue defects and progressive neurological degeneration, with death in early childhood[14]. A similar flaw of copper metabolism results in copper accumulation in cultured cells[15,16] and copper maldistribution in tissues of the Menkes infant[14]. Both mouse and human disorders have been mapped to homologous regions of the X chromosome near the phosphogylcerate kinase (*Pgk1/PGK1*) locus[17,18]. These parallels have led several authors to propose that Menkes disease and the mottled mouse phenotypes result from defects in homologous genes[4,19]. The recent identification of the Menkes disease gene (*MNK*)[20–22], which encodes a putative copper-transporting ATPase, allows a direct test of this hypothesis.

In this report, we identify the mouse homologue of the Menkes disease gene, demonstrate that it maps to the mouse X chromosome near *Pgk1*, and characterize two different defects in the expression of the mouse homologue in the Mo^{blo} and Mo^{dp} hemizygotes. These findings prove that the mottled and Menkes disorders result from mutations in homologous genes and suggest that further studies on the mouse will provide insights into Menkes disease.

Isolation and sequence analysis

We isolated murine cDNA clones by virtue of their homology to Mc1, our cDNA for the Menkes disease gene[20]. A combination of cDNA library screening with human Mc1 probes, RT-PCR amplification of murine RNA with human Mc1 primers and the 5' RACE procedure identified cDNA clones spanning the entire coding region of the mouse gene. The cDNA sequence of the overlapping clones consisted of 4,889 basepairs (bp) including a 4,473-

bp open reading frame encoding a predicted 1,491-amino-acid protein (Fig. 1). The sequence, which has been deposited in Genbank (accession no. U03434), includes an 82 nucleotide 5' untranslated region and 334 nucleotides of the 3' untranslated region. The open reading frame in the murine sequence is 88% identical to that of the human and the derived amino acid sequence is 89% identical.

Comparative database analysis revealed that the translated mouse cDNA is most closely related to the human gene product, as expected. Like the predicted human protein[20], the derived mouse amino acid sequence contains the signature domains of a P-Type ATPase[23] and six N-terminal metal binding motifs. The next most similar protein is *Enterococcous hirae* CopA followed by *E. hirae* CopB, both of which are proven copper-transporting ATPases[24].

The six metal binding motifs depicted in Fig. 1 indicate a minimum motif defined by homology to motifs in other metal binding proteins[25]. The mouse, human and copA motifs are more related to each other than to other metal binding motifs (data not shown), and extend beyond the minimum motif. We postulate these motifs may have specificity for copper. We note that the regions between each metal binding motif, except motifs 5 and 6, in the mouse and human contain several proline residues which may allow for flexibility in these spacers (Fig. 1).

Hydropathy analysis[26] of the mouse protein and reassessment of the human amino acid sequence suggest that the human and murine proteins contain eight transmembrane domains instead of the six originally proposed for human[20]. Regions including the proposed transmembrane domains are indicated by boxes in Fig. 1.

Expression in mouse parallels human

The mouse cDNA was hybridized to a northern blot containing polyA$^+$ RNA from a variety of mouse tissues (Fig. 2). The size of the mouse mRNA is ~8.3 kilobases (kb), slightly smaller than the corresponding human

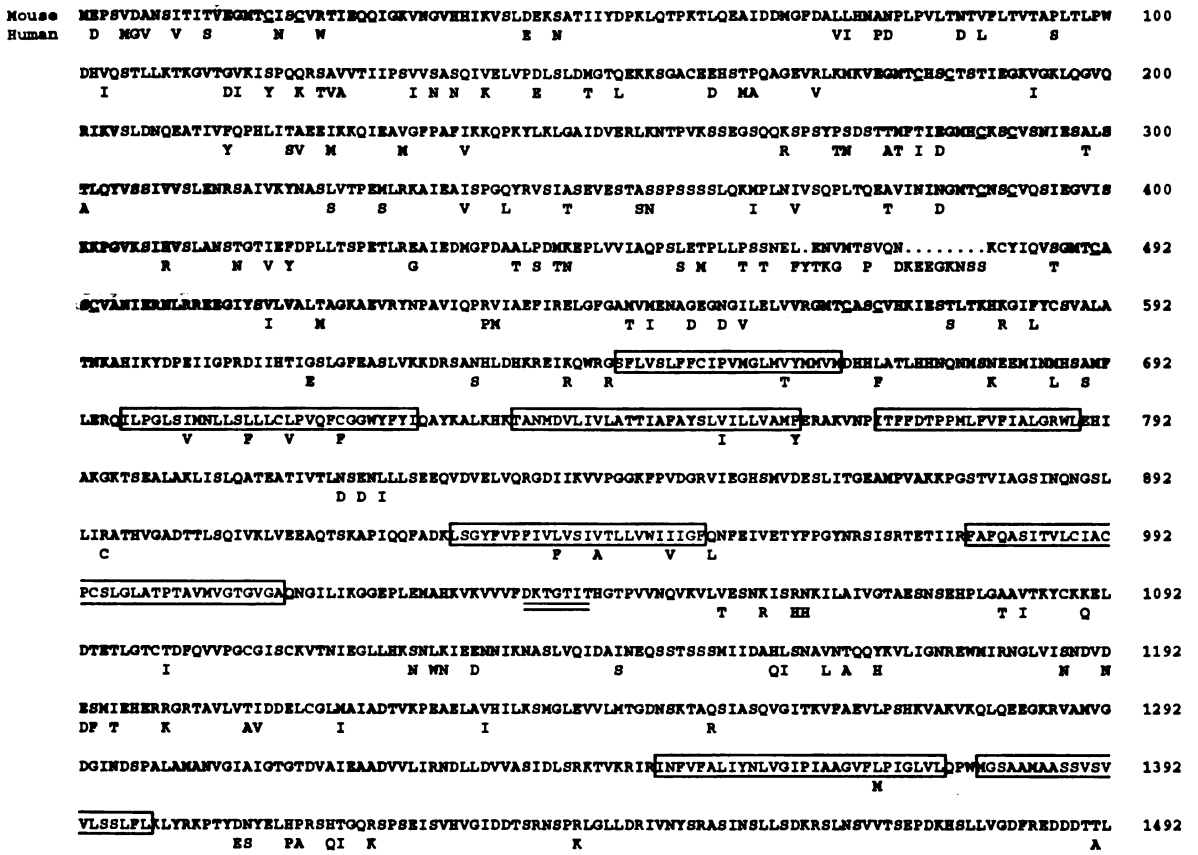

Fig. 1 Comparison of the derived amino acid sequences for the murine and human copper-transporting ATPases. The murine protein sequence is shown on top. Any amino acid which differs in the human is shown in the line below. The murine protein is eight amino acids shorter than the human and a gap was introduced in the mouse sequence. The numbering on the right side of the figure corresponds to the mouse sequence. The six metal binding motifs are shaded with the invariant cysteines underlined. Eight hydrophobic domains which may represent transmembrane domains are boxed. DKTGTIT, a signature sequence for P-type ATPases, is underlined twice.

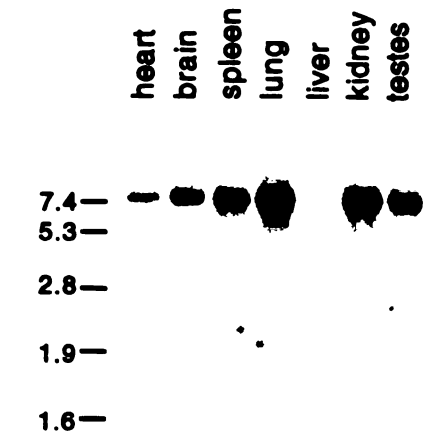

Fig. 2 Tissue distribution of expression of the murine copper-transporting ATPase gene. The mouse tissue northern blot was hybridized to a partial human Mc1 probe. An ~8.3 kb transcript is seen in all tissues except the liver.

transcript. As the murine open reading frame is only nine codons shorter than the human, this finding suggests that an additional ~3.4 kb of 3' untranslated sequence remains to be identified for the murine cDNA. As in the human, the mouse gene is expressed in the heart, brain, kidney, spleen, lung, pancreas and placenta, whereas the liver has very low levels of the transcript. This expression pattern is consistent with the disease manifestations in the mottled mouse[5,8].

Mapping the murine gene

One criterion for establishing whether the isolated mouse gene is the mottled gene is to determine if it maps to the mottled locus on the X-chromosome, near the *Pgk1* locus. Since the *MNK* gene maps within 100 kb centromeric of *PGK1* (refs 20–22, 27), we tested two previously isolated mouse *Pgk1* containing yeast artificial chromosomes (YAC) clones for the presence of the corresponding mouse gene. All of the restriction fragments detected by the mouse cDNA probe in genomic DNA were present in the two YAC clones (data not shown). These results prove that the mouse copper-transporting ATPase gene is located in a site appropriate for the mottled locus.

Mouse gene abnormalities in mottled alleles

To test whether mutations in the murine gene give rise to the mottled phenotype, we analysed RNA and DNA prepared from tissues or cell lines of hemizygotes for five different mottled alleles. Northern, and Southern blots of DNA digested with three different enzymes, were hybridized with the mouse cDNA probe. No gross abnormalities were detected in the Mo^{prw}, Mo^{ml} and Mo^{br} males, but striking changes were observed in the Mo^{dp} and Mo^{blo} males.

Mo^{dp} hemizygote embryonic fibroblasts do not express the murine copper-transporting ATPase gene, while cultured cells from normal male and female littermate embryos do (Fig. 3*a*). Southern blot analysis with a probe from the central portion of the mouse cDNA (nts 1819–4160) revealed altered and absent restriction fragments in the genomic DNA of the Mo^{dp} hemizygote (Fig. 3*b*). As probes corresponding to 5' and 3' regions of the cDNA detected no alterations (data not shown), we conclude that a small interstitial deletion in the mouse gene is the Mo^{dp} mutation. This interstitial deletion probably leads to an unstable mRNA.

The Mo^{blo} hemizygote also shows abnormal expression of the mouse gene (Fig. 3*c*). Three transcripts are detected in the Mo^{blo} brain: decreased levels of a normal sized transcript and two additional larger transcripts. No gross genomic rearrangements were detected by Southern blot analysis in the Mo^{blo} mice (data not shown) suggesting a possible splicing defect as the basis of the Mo^{blo} allele.

Discussion

We conclude that the murine homologue of the Menkes disease gene (*MNK*) is the mottled (*Mo*) gene. The murine copper-transporting ATPase gene encodes a protein with 89% identity to the Menkes disease gene product and is expressed in the same tissues as the human gene. Both genes map to homologous regions of the mouse and human X chromosome near the *Pgk1/PGK1* locus at the established position of the mottled locus. And most significantly, we demonstrate a mutation in the mouse gene in Mo^{dp} mice and altered expression in Mo^{blo} mice.

In addition to the common copper-transporting defect, a remarkable concordance of pathologic findings is seen in Menkes disease patients and the mottled mice hemizygotes. As a group, the mottled alleles manifest all the specific pathologic changes in Menkes disease that are presumed to result from copper enzyme deficiencies[3,14]. For example, lysyl oxidase deficiency likely results in the skeletal and arterial pathology noted in both species[12,28]. Deficient activity of tyrosinase underlies the hypopigmentation in Menkes patients and mottled hemizygotes as well as the variegated coat in mice or skin colour in man of heterozygotes of both species[29,30]. Other changes, such as a unique cerebellar pathology and hair shaft deformities have not been attributed to a specific enzyme deficiency but occur in both disorders[31–33].

The range in phenotypes in mottled mice suggest that defects in the Menkes disease gene may result in a similar spectrum of phenotypes. Patients with milder and atypical forms of Menkes disease have been reported[34–36], and particular murine alleles seem to mimic these disorders[14]. For example, Mo^{blo} mice may provide a model for X-linked cutis laxa, a disorder of copper transport and a likely allele of Menkes disease[37]. X-linked cutis laxa patients have multiple bony abnormalities, hyper-extensible joints, lax skin, and bladder diverticulae[38]. Mo^{blo} males similarly exhibit profound connective tissue abnormalities and neither the human or the mouse mutant displays severe neurological problems[3]. The finding of altered forms of mRNA in the Mo^{blo} mouse, and the similarities in phenotype, suggest that study of this allele will prove valuable in the understanding of X-linked cutis laxa.

Although Menkes and mottled disorders are remarkably similar, we note that partial deletions of *MNK/Mo* affect man and the Mo^{dp} hemizygote differently. A deletion of the *Mo* gene in the Mo^{dp} hemizygote results in reproducible prenatal lethality, whereas the ~20% of Menkes patients with deletions of *MNK* are live born[20,22]. This difference suggests that other genetic factors or species differences in copper metabolism may influence fetal survival.

Studies of the mottled mouse suggest therapeutic

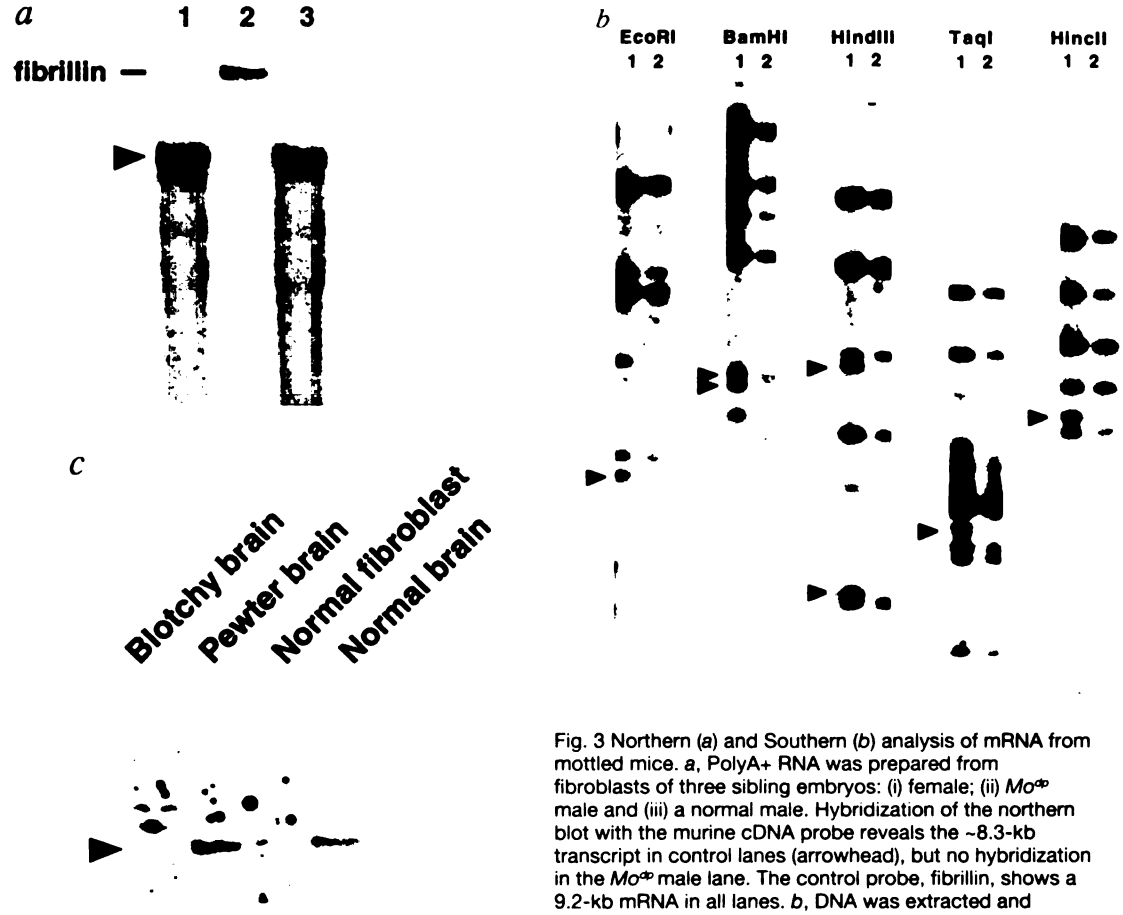

Fig. 3 Northern (*a*) and Southern (*b*) analysis of mRNA from mottled mice. *a*, PolyA+ RNA was prepared from fibroblasts of three sibling embryos: (i) female; (ii) Mo^{dp} male and (iii) a normal male. Hybridization of the northern blot with the murine cDNA probe reveals the ~8.3-kb transcript in control lanes (arrowhead), but no hybridization in the Mo^{dp} male lane. The control probe, fibrillin, shows a 9.2-kb mRNA in all lanes. *b*, DNA was extracted and digested from the female control (lane 1) and Mo^{dp} male (lane 2) embryonic fibroblasts. The Southern blot was hybridized to the mouse cDNA probe. Arrows indicate bands in the control lane that are missing or altered in the Mo^{dp} lane; *Eco*RI, 2.1 kb; *Bam*HI, 3.6 kb and 3.8 kb; *Hin*dIII, 1.2 kb and 3.9 kb; *Taq*I, 1.7 kb; *Hin*cII, 3.1 kb. *c*, A northern blot of polyA+ RNA prepared from normal and mutant fibroblasts and brain tissue was hybridized to the mouse Mc1 probe. The normal ~8.3 kb transcript is arrowed. Two larger transcripts are also observed in Mo^{blo} brain.

strategies for Menkes disease. Although copper therapy has been ineffective in the treatment of severe Menkes infants[39], copper supplementation in neonatal Mo^{br} and Mo^{ml} males has proven effective[40,41]. For these mice, a critical window of postnatal age seven to ten days has been established when copper therapy can prevent the neurologic disease, growth retardation and lethality[40,42]. Detailed studies in mottled mice also suggest that lipophillic cuprous-complexes are more effectively delivered across the blood brain barrier than are cupric, hydrophillic complexes[43,44]. The 7–10 day postnatal mouse brain corresponds to about the same developmental stage as the middle third trimester in the human fetal brain. Therefore, the treatment in Menkes disease may need to be initiated prenatally with one of these more effective agents.

Menkes disease and mottled mice may provide insight into other disorders of copper metabolism or copper enzymes. The existence of inherited disorders as diverse as Wilson disease, Indian childhood cirrhosis, inherited copper toxicosis of dogs and the LEC rat and the toxic milk mouse suggest that the system for copper homeostasis is complex[45]. Perturbation of components of this system may also contribute to the pathogenesis of inherited defects of copper enzymes such as amyotrophic lateral sclerosis, mitochondrial myopathies and oculocutaneous albinism which result from defects in the copper enzymes superoxide dismutase[46], cytochrome oxidase[47] and tyrosinase[48], respectively.

Methodology

Animals. The Mo^{blo} and Mo^{brs} heterozygotes were purchased from Jackson Laboratory and were mated with C57BL/6J males to yield affected male offspring. The Mo^{dp} heterozygotes were provided by Roland Quinney (MRC, Didcot, UK) and mated with C57BL/6J males. Mo^{ml} hemi- and heterozygote animals were received from Hiroko Kodama at Teikyo University, Tokyo.

Cells and cell culture. Cultured fibroblasts were prepared by standard techniques from various tissues from mice with different mottled alleles and propagated in Dulbecco's modified Eagles H21 medium plus 10% fetal calf serum[49]. Embryonic cultures from 14 d.p.c. embryos from the mating of a Mo^{dp} heterozygous female and a C57BL/6J male were established by trypsinizing the embryos at 4 °C overnight and plating in H21 DME plus 10% fetal calf serum. PCR using *sry*-specific primers was used to determine the sex of the embryos. The male and control female littermate embryonic cultures were then tested for copper accumulation by atomic absorption spectrophotometry of whole cell extracts as described[49]. Mo^{dp} hemizygotes cell lines were identified which accumulated 8-fold more copper than litter-mate controls.

Isolation of cDNA clones. An adult Balb/c mouse brain cDNA library was purchased from Stratagene and 10^6 plaques were screened with a partial human Mc1 cDNA probe hybridized at 50 °C. The isolated positive phage clones were digested with *Eco*RI and the fragments cloned into SK⁺ Bluescript (Stratagene). The largest clone, 3,071 bp includes 2,737 bp of open-reading frame and 334 bp of the 3' untranslated region. This clone represents nucleotides 1819–4889 in the GenBank sequence.

In order to identify the 5' end of the mouse gene, poly A⁺RNA from C57BL/6J fibroblasts was reverse-transcribed using random primers and the Invitrogen cDNA Cycle Kit. The resulting cDNA was PCR amplified using three different nested sets of oligonucleotide primers to the human and murine gene. The products were cloned into the Srf vector (Stratagene). These clones represent nucleotides 1110–1819 of the sequence. A BRL 5' RACE kit was used to isolate the remaining 5' region of the mouse cDNA.

cDNA sequencing. Sequencing of double-stranded cDNA clones was performed with a USB Sequenase Version 2 kit using T3 and T7 as well as internal primers.

RNA preparation and northern blot analysis. Poly A⁺RNA was isolated from 10^7 cultured cells or a 0.1 g sample of tissue using a Pharmacia QuickPrep Micro RNA Purification Kit. Approximately 2 μg of mRNA was loaded onto a 0.8% agarose gel and blotted onto Amersham Hybond nylon filters. In addition, a mouse multiple tissue northern blot was purchased from Clonetech. These blots were hybridized with partial mouse or human Mc1 probes at 60 °C.

Mouse YACs. Two mouse *Pgk1*-containing YACs, C15.F1 and C96.G2, were provided by the Princeton mouse YAC library. The YACs were grown on YPD medium and DNA was prepared as described[50].

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Chapter 5

Towards a localization of the Menkes disease gene product

The predicted amino acid sequence of the Menkes and Mottled disease genes predict integral membrane P-type ATPases which transports copper across cellular membranes. Biochemical analysis of cells from Menkes patients and Mottled mice suggest a copper transport defect in the export of copper from cells. The location of this defect in copper export is not known although there is support for a subcellular transport defect in copper transport into an intracellular compartment or organelle. In this chapter, I will describe the efforts towards determining the subcellular location of the Menkes disease putative copper transporting ATPase. Two approaches were taken in this endeavor: localization by utilizing polyclonal antibodies prepared to the Menkes protein, and localization by using epitope tagging of a complete open reading frame cDNA. Although the subcellular localization has not been determined at the writing of this thesis, significant progress has been made toward this end. I will first describe the biochemical studies which bear on the question of cellular locale, then the work towards the production of a polyclonal antibody specific for the Menkes copper transporting ATPase, and finally describe the construction of the Menkes cDNA and the expression of an epitope tagged protein in stably transfected cell lines.

Predicted cellular location of the Menkes disease gene product

Biochemical characterization of Menkes and Mottled cell lines suggest a subcellular defect in copper export rather than a plasma membrane defect. The increased copper content in Menkes/Mottled cells accumulates preferentially in the cytoplasm bound to metallothionein. In spite of overall increased copper in Menkes/Mottled cells, decreased copper content in

mitochondria and lysosomes has been demonstrated in mutant cells by biochemical measurements (Kodama et al., 1989) and electron microscopy (Kodama et al., 1993). Studies of the distribution of copper and zinc found coordinate increase in the copper content of particulate and cytosolic fractions as copper content increased in normal control cells cultured in excess copper (Packman et al., 1987). However, in Menkes cells, over a similar range of cytosolic copper concentrations particulate copper content remained significantly lower than control cells, while zinc distribution was identical in both. A defect in copper transport into an intracellular membrane compartment could provide an explanation for these findings.

Differential response of copper enzymes to copper supplementation in patients with Menkes disease and Mottled mice and in mutant cultured cells similarly support a defect in copper translocation across intracellular compartments. Copper supplementation by parenteral injection restores the activity of the cuproenzymes tyrosinase, and cytochrome c oxidase in mottled mutants. Copper prevents seizures and perinatal death of males carrying the Mo^{br} or Mo^{ml} alleles but had no effect on the connective tissue disease of the Mo^{blo} mouse (Mann et al., 1979; Nagara et al., 1981; Royce et al., 1982; Shiraishi et al., 1988). These studies parallel observations on copper therapy in patients with Menkes disease: copper supplementation of patients born prematurely or identified at birth ameliorated many symptoms of the disorder, but connective tissue defects persisted (Christodolou et al., 1994; Danks, 1994). The connective tissue disease of these disorders has been ascribed to reduced production and activity of the cuproenzyme lysyl oxidase, and suggests a particular sensitivity of lysyl oxidase in patients with Menkes disease and Mottled mice.

Cell culture studies also demonstrate that lysyl oxidase responds differently to copper supplementation than do other cuproenzymes in mutant cells. Although the activity of all tested copper enzymes is decreased in tissues from patients with Menkes disease or mottled mice (Hunt, 1977; Hunt and Johnson, 1972; Phillips et al., 1986; Rezek and Moore, 1986; Rowe et al., 1977; Royce and Steinmann, 1990; Seki et al., 1989; Sparaco et al., 1993; Yoshimura, 1988), activity of some intracellular copper enzymes can be restored by cell culture in presence of exogenous copper. In studies in blotchy cultured fibroblasts, copper is available for incorporation into cytoplasmic superoxide dismutase (Packman et al., 1984)while the specific activity of secreted lysyl oxidase is distinctly reduced in Menkes disease cells (Kuivaniemi et al., 1985). Copper supplementation was ineffective in restoring lysyl oxidase activity in *Mo^{blo}* extracts (Royce et al., 1982) or OHS or Menkes cell line preparations (Kuivaniemi et al., 1985). The resistance of lysyl oxidase to in vitro and in vivo copper therapy may reflect the intracellular site of lysyl oxidase synthesis and together with biochemical studies provide clues into the site of function of the Menkes/Mottled copper transporting ATPase. Lysyl oxidase in contrast to other cuproenzymes is a secreted enzyme (Kuivaniemi et al., 1985). The Menkes/mottled copper transporting ATPase may be required in the transfer of copper to the specific cell compartment in which holoenzyme is formed. In contrast, other cuproenzymes may be located in the cytoplasm or cellular compartments accessible to available copper, without the requirement of an additional transport step mediated by the Menkes/mottled proteins. Cytosolic copper utilization may be adequate in Menkes/Mottled cells, while delivery of copper to proteins or enzymes in other cell compartments is deficient.

The biochemical data and mutant phenotypes, therefore, suggest that the Menkes/Mottled disease gene products may be important for copper delivery to the mitochondria and lysosomes and essential for copper delivery to the secretory path organelles such as the endoplasmic reticulum or Golgi. The intracellular localization of the Menkes copper transporting ATPase protein may reflect this requirement an therefore may expressed in all of these compartments, mitochondria, lysosome and endoplasmic reticulum, or alternatively a intracellular compartment responsible for delivery of copper to all these organelles.

Polyclonal antiserum to Menkes disease gene product fusion protein

Two approaches were attempted to produce polyclonal antibodies to the Menkes copper transporting ATPase in order to resolve the question its intracellular location. One approach has been to express a 480 amino-acid peptide corresponding to the amino-terminal putative copper-binding domain as a glutathione S-transferase (GST) fusion protein (Gearing et al., 1989) for use as an immunogen. This region was chosen because it was unique to the Menkes copper transporting ATPase at the time of the work, it is predicted to be exposed in a proposed model of the protein thus facilitating immuno-localization, and is hydrophilic overall therefore likely resulting in a soluble and immunogenic fusion protein. The pGEX vector system was used to express the N-terminal fragment in E. coli as a glutathione S-transferase (GST) fusion protein (Gearing et al., 1989). A 1440 bp EcoR1 fragment of the composite Menkes cDNA which encodes a 480 amino acid peptide from amino acid 1 to 480 and containing the first four metal binding motifs was cloned in frame into the pGEX3 vector (Pharmacia). A factor X_a cleavage site allows separation of the 480 aa peptide from the GST portion of

the fusion protein by protease treatment (Smith and Johnson, 1988). Fusion protein was expressed in *E.coli* SURE cells (Stratagene) and purified from lysates with glutathione agarose beads (Sigma). The 480 amino-acid peptide was released by Factor X_a cleavage. Rabbit polyclonal antibodies against the peptide were prepared by ImmunoDynamics, Inc. in two different rabbits and their characterization is described below.

Anti-peptide antisera

A second approach was to produce anti-peptide antibodies. Three peptides corresponding to distinct regions of the copper transporting ATPase were used to generate antibodies. Each of the peptides, 19-32 amino acids in length, are hydrophilic and were predicted to be immunogenic by computer analysis. Peptides were chosen which were not within the consensus sequences common to all cation ATPases, but rather in selected regions shared uniquely by Menkes protein and the *E.hirae* CopA protein, its closest homologue at the time of the work. The three peptides correspond to: the first metal binding motif (amino acid 14 to 47 in the predicted Menkes protein), a predicted cytoplasmic region near the phosphatase domain (amino acids 936-955), a region near the ATP-binding domain (amino acid 1299-1321). This last peptide was chosen in consultation with Dr. David MacLennan's laboratory (Toronto) as peptides in a similar relative location (but of different sequence) were found to be immunogenic in the sarcolemmal calcium-transporting ATPase.

Analysis of antisera

Each of the antisera was tested for its sensitivity and specificity. One criteria for evaluating whether an antiserum recognized the Menkes protein

was to test whether it recognized a protein of the appropriate predicted size (1500 amino acids for an expected size of ~165 kilodalton excluding glycosylation) and expected characteristics. The Menkes protein should be present in normal cell extracts but not in extracts from patients with Menkes disease in which severe mutations prevent expression of the Menkes disease gene. Fibroblasts and lymphoblasts were chosen as the tissues for study as cells of this type from patients with Menkes disease express the cellular phenotype of copper accumulation (Danks, 1989; Herd et al., 1987), and therefore normal cells of this type likely express the Menkes protein.

A ~200 kd protein in normal and Menkes cells

A ~200 kd protein is detected by polyclonal antisera the N-terminal fusion protein and the N-terminal peptide. A antiserum (one of two prepared) to the N-terminal fusion protein and a antiserum (also one of two) reacted strongly with the fusion protein and the purified 480 aa peptide at dilution of up to 1/2500 (data not shown). Western analysis of cell extracts prepared by a variety of means demonstrated immunoreactivity with a protein of ~200 kd in normal cells, slightly larger than expected size of the Menkes protein, as well as several smaller molecular weight proteins with these antiserum. The anti-peptide serum gave greater immunoreactivity under the experimental conditions used (Figure 15). Pre-immune sera gave no detectable reaction with the ~200 kd protein. However, under the conditions tested, the antisera reacted to proteins of identical size in extracts from cells of the Menkes translocation patient, C.G., which do not express any detectable mRNA and therefore should not express the Menkes protein. Extracts from other Menkes patients, some with similar severe mutations, gave identical results. It should be noted, at this point, that an additional antisera prepared to a GST-

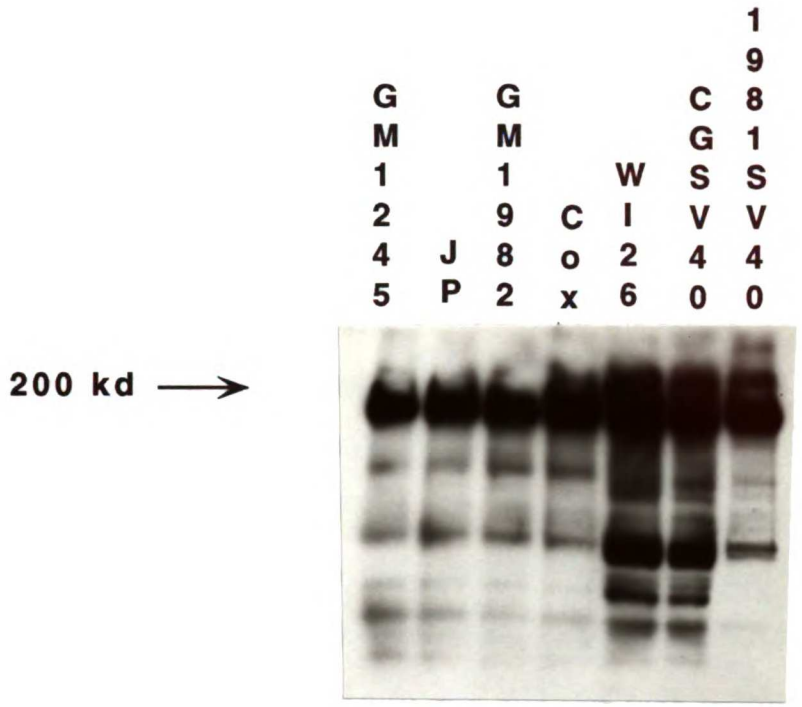


Figure 15: Antiserum to metal binding motif peptide identifies a prominent ~200 kd protein in normal and Menkes patient. GM1245, J.P. and GM1982 are lymphoblast cell lines from Menkes patients, Cox is a normal lymphoblast control, WI26 is a normal SV40 transformed fibroblast cell line, while C.G. SV40 and 1981 SV40 are SV40 transformed fibroblast cell lines from patients with Menkes disease. A ~200 kd band is evident in all cell lines, including the Menkes cell lines such as C.G. SV40 with a demonstrated lack of expression of the MNK mRNA. Protein lysates were prepared by lysis of culture cells at 10^7 cells/ml in NP40 lysis buffer(140 mM NaCl, 50mM Tris-Cl pH 7.5, .5% NP40, 1mM EDTA, 10% Glycerol). Twenty lambda of lysate was added to twenty lambda 2X SDS loading buffer and loaded onto a 4-15% SDS protein gel. The gel was run at 100V for 1hr 30 min and semidry blotted to a supported nitrocellulose membrane. The blot was blocked in 5% NFDM(Non-Fat Dry Milk) in TBST(100 mM NaCl, 10 mM Tris-HCl ph 8.0, .1% Tween-20) for 3 hrs. Antiserum to the first metal binding motif peptide(1703) of the Menkes protein was added to a dilution of 1/1000 in TBST and incubated at 4°C overnight , rocking. The blot was washed four times in TBST, blocked 30 min in 5% NFDM in TBST, and incubated with a 1/1000 dilution of Donkey anti-rabbit IgG-HRP(Amersham) for 1hr at 22°C, and washed washed four times in TBST. ECL detection reagents (Amersham) and autoradiography was used to visualize immunoreactive proteins.

fusion protein containing the fifth and six metal binding motifs by another laboratory (J.Gitlin) and generously provided to us gave generally stronger signals but with identical results, a strong ~200 kd protein as well as smaller bands which are present in normal and Menkes cell lines.

Several explanations are possible, first the antisera may not recognize the Menkes protein specifically and the immunoreactivity to the fusion protein is spurious, second the antisera does recognize the Menkes protein but also cross reacts with a protein of the identical or very similar size , or third the antisera contains a combination of specific antibodies and non-specific antibodies which coincidentally recognize a protein of the same molecular weight, or fourth the western blot analysis may require further optimization. The discovery of the Wilson disease gene which encodes a protein homologous to the Menkes disease protein and of very similar molecular weight provides a precedent for multiple metal transporting ATPase in mammalian cells (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1993). The Wilson disease gene and therefore the protein, however, is reportedly not expressed in fibroblasts and lymphoblasts, and therefore does not provide an explanation for the presence of immunoreactive material in the cell lines from the null mutant Menkes cell lines. The complex task of cellular metal (copper or other metals, such as zinc) import, export, and intracellular transport may well utilize additional metal transporting ATPases and such a protein present in fibroblast and lymbloblast cell lines may provide an explanation for the results with the antisera.

Similar analysis of the other antipeptide antisera, to the amino acid 936-955 peptide and to the 1299-1321 peptide showed considerable variation in the

immunoreactivity of the antisera. Multiple different proteins were detected by the antisera in westerns. None of these proteins was of the expected size of the Menkes protein and were found in both Menkes and normal cells (data not shown).

Affinity purification is unsuccessful

Affinity purification of the antisera did not increase the specificity of the antisera. Antisera to the N-terminal fusion protein peptide were affinity purified on a antigen column prepared with the purified 480 amino acid peptide. Affinity purification of the antisera resulted in no detectable change in the specificity of the sera, and there was a consistent decrease in reactivity of different batches of eluted antisera. The antisera to the first metal binding motif was also purified on a 480 amino acid N-terminal peptide column and will henceforth be designated 1703 AP. The resulting affinity purified antisera was tested by western analysis. Similarly, antisera to the peptides were affinity purified by preparation of peptide columns, and binding and elution of specific antisera. The affinity purified antipeptide antisera in general detected fewer protein products on western analysis, the affinity purified α-Mc1 936 antisera detected a prominent less than 46 kd protein, the affinity purified α-Mc1 1299 antisera detected a ~80kd protein, and the 1703 AP antiserum detected a prominent ~100 kd protein.

A ~200 kd protein in normal but not Menkes cells

Preliminary results indicate that variation of the western blotting conditions may increase the specificity of the antiserum. The addition of Triton -X and SDS to the wash buffer of the western blots resulted in marked loss of immunoreactivity. However with the antiserum from the laboratory of J.Gitlin, prepared to the 5th and 6th metal binding motifs, a 200 kd protein

was detected in a normal cell line but not in a C.G. translocation cell line (Figure 16). Unfortunately, the signal from the antiserum prepared in this laboratory to the first metal binding motif peptide in these conditions was poor, an no indication of specificity was seen. It should be noted that although this result was repeated, there were difficulties in consistent replication, and should be considered preliminary. It is not clear what differences in experimental conditions were responsible for this variations, although variables such as temperature and exact buffer composition of the washes may be contributory

No definitive localization

The polyclonal antisera do not provide definitive identification of the protein encoded by the Menkes gene. An immunoreactive protein or proteins of ~200 kd is present in cellular extracts with antisera prepared to the N-terminal first metal binding motif peptide, to a fusion protein including the first through fourth metal binding motifs, and to a fusion protein including the fifth and six metal binding motifs. The antisera react to a ~200 kd protein in both normal and null mutant cell lines under non-stringent western blotting conditions. One antiserum, to the fifth and six metal binding motifs, may identify, utilizing certain more stringent western blotting conditions, a specific protein in normal cells which is not present in Menkes cells. Additional work clearly needs to be done to utilize these antisera for immunolocalization and possibilities are discussed in the next chapter.

Construction of epitope tagged Menkes cDNA

A complete Menkes open reading frame cDNA was constructed and expression demonstrated in stably transfected cell lines. Two rationales

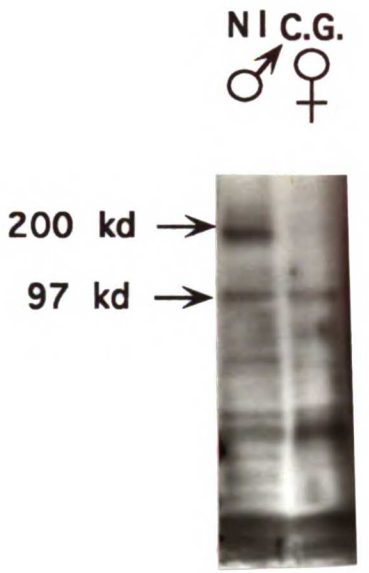
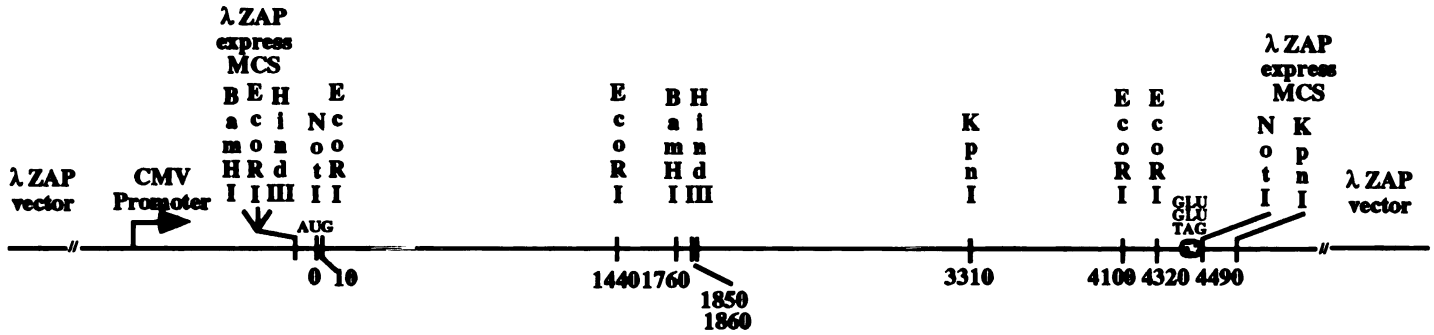


Figure 16: Antiserum to fusion protein containing 5^{th} and 6^{th} metal binding motifs peptide identifies a prominent ~200 kd protein in normal but <u>not</u> in Menkes patient. Normal (NI) lymphoblast control is Cox cell line from the Gitschier laboratory, while C.G. is CG UML-3 a lymphoblastoid cell line established from the translocation patient (A gift of T.Glover). A ~200 kd band is evident in the normal control which is not present in C.G. Protein preparations were by lysis of approximately equivalent amounts of cells in 2X SDS lysis buffer(125 mM Tris-Cl pH6.8, 20 % glycerol, 4% SDS, 200 mM DTT) at 95°C for 5 min. Protein concentration were determined by Biorad protein quantitation reagents and 50 µgs of total cellular protein were loaded per lane on a 4-15% SDS gradient gel(Biorad), run at 100 V for 1.5 hrs and semidry blotted onto supported nitrocellulose. The blot was blocked in 5% BSA in TBST(100 mM NaCl, 10 mM Tris-HCl ph 8.0, .1% Tween-20) for 3 hrs. The antiserum to the 5^{th} and 6^{th} metal binding motifs of the Menkes protein(A gift of J.Gitlin)was added to a dilution of 1/1000 in TBST+5%BSA and incubated at 4°C overnight , rocking. The blot was washed four times in 100 mM NaCl, 10 mM Tris-HCl ph 8.0, .5% Triton-X, .1% SDS; blocked 30 min in 5% BSA in TBST; and incubated with a 1/1000 dilution of Donkey anti-rabbit IgG-HRP(Amersham) for 1hr at 22°C, and washed washed four times in 100 mM NaCl, 10 mM Tris-HCl ph 8.0, .5% Triton-X, .1% SDS. ECL detection reagents (Amersham) and autoradiography was used to visualize immunoreactive proteins.

underlay the work: complementation studies and immunolocalization of the Menkes protein. Although numerous mutations documented in the candidate gene isolated by our group suggest it is the Menkes gene, complementation of the cellular phenotype of copper accumulation by expression of the cDNA in mutant cells would provide direct confirmation of its role in Menkes disease. As well epitope tagging of the expressed protein by modification of the complete ORF cDNA could provide an alternative means to determine the cellular location of the protein.

Assembly of cDNA

A complete 4.6 kb open reading frame (ORF) cDNA modified to contain the sequence for an epitope tag was assembled stepwise from previously isolated partial cDNA clones and newly isolated RT-PCR products. Advantage was taken of convenient restriction sites in the composite Menkes cDNA to link individual cDNA pieces (Figure 17). A three prime piece was assembled first. A cDNA piece stretching from a unique Kpn1 site at position 3310 in the Menkes cDNA to the stop codon at position 4490 was PCR amplified from a previously isolated cDNA clone. PCR primers specific to Menkes cDNA were used however the three prime end was modified to include sequences specifying a "Glu-Glu" epitope tag in frame with the protein. The "Glu-Glu" epitope is an eight amino acid tag for which a specific monoclonal antibody exists (Grussenmeyer et al., 1985). A number of proteins have recently been localized by this method including a subunit of a G protein (Levis and Bourne, 1992), a GTPase activating protein (Rubinfeld et al., 1991), as well as mutant vasopressin receptors (Pan and Gitschier, unpublished data). This three prime clone was ligated to another cDNA piece covering from the unique BamHI site at position 1760 to the Kpn1 site at 3310



	Sense Orientation Restriction Fragments	Anti-Sense Orientation Restriction Fragments
EcoRI	20, 220, 1430, 2660	180, 220, 1430, 2660
BamHI	1760	2730
HindIII	1850	2630
KpnI	1180	3310

200 bp

Figure 17: Expected restriction size fragments of MNK ORF cDNA cloned into λ ZAP express vector. Restriction sites in MNK cDNA were utilized in the assembly of a complete ORF cDNA and allow analysis of λ ZAP clones. The numbering refers to the position relative to the AUG codon in the MNK cDNA. The MNK cDNA was cloned into the Not1 site in the multiple cloning site of the λ ZAP express vector. Sequences encoding a "Glu-Glu"epitope tag were added to the three prime end of the cDNA. The orientation of the cDNA relative to λ ZAP multiple cloning site (MCS) determines the fragment sizes from restriction digestion with BamHI, HindIII, and KpnI. The cDNA above is presented in the "sense" orientation such that the CMV promoter directs the production of transcript encoding the 1500 aa Menkes protein with the "Glu-Glu" epitope tag at the C-terminus.

which was also prepared from a previously isolated cDNA clone. The resulting product from the BamHI to the stop codon was cloned and is referred to as the three prime clone. The five prime end from the AUG at bp 0 to the BamHI site at position 1760 was isolated by direct RT-PCR of normal fibroblast mRNA with appropriate Menkes specific primers and cloned. Both of these two pieces, the three prime clone and the five prime clone, together comprise the entire ORF cDNA were extensively characterized by restriction enzyme digestion and sequence analysis of the junctions and ends of the cDNA pieces. The two pieces were determined to likely faithfully represent the Menkes cDNA.

Complete cDNA in plasmid vector rearranges in *E.coli*

Significant difficulties were encountered in attempting to propagate the complete cDNA in a standard plasmid cloning vector in *E.coli.* Multiple attempts at the cloning of the 4.6 kb ligation product of the two pieces did not recover a complete cDNA. The starting products or multiple different rearranged products were found instead. Filter hybridization screening with probes specific to each end did identify some clones with sequences from both cDNA pieces. Restriction analysis of these clones indicated significant rearrangement as before. This phenomenon was independent of the orientation of the cloned cDNA in the plasmid vector. *E.coli* strains which propagate the cloning vector at low copy (Able C and K from Stratagene) did not facilitate recovery of a complete cloned product. It is not clear what is the cause of this cloning difficulty. One possibility is that the complete cDNA in contrast to the individual pieces is toxic to *E.coli.* Anecdotal evidence from other workers in the field (J.Mercer) who reported similar cloning difficulties supports such a possibility.

Cloning of complete cDNA in lambda

An alternative cloning vector, Stratagene λZAP express™ vector, was successfully used to obtain a complete Menkes ORF cDNA. The ligated ~4.5 kb product of the two individual five prime and three prime pieces was cloned into the λZAP express™ vector. Hybridization screening with the five prime and three prime probes identified multiple clones containing sequences hybridizing to both. Restriction digests of these clones was consistent with full length cDNA (Figure 18). Further analysis of seven clones revealed three sense and four antisense clones (Figure 18).

No detectable mutations in complete cDNA

One sense clone was analyzed for point mutations. To determine whether any point mutations were introduced during the cloning process, the chemical cleavage technique (Cotton et al., 1988) was use to analyze one selected sense clone. Briefly the chemical cleavage mutation detection technique is based on the ability of the osmium tetroxide and hydroxylamine to modify mismatched bases in DNA heteroduplexes between control and test DNA. Piperdine treatment of the heteroduplex results in a cleavage of one strand if there is a modified base, which can be detected by denaturing electrophoresis of the labeled reaction products. The chemical cleavage technique is considered the most accurate of the mutation detection procedures available, with detection of virtually 100% of mutations (Condie et al., 1993), and has been use successfully for a number of disorders including Menkes disease (Das et al., 1994; Grompe et al., 1989; Montandon et al., 1989; Nadal and Baerlocher, 1988; Naylor et al., 1993). The Menkes ORF cDNA

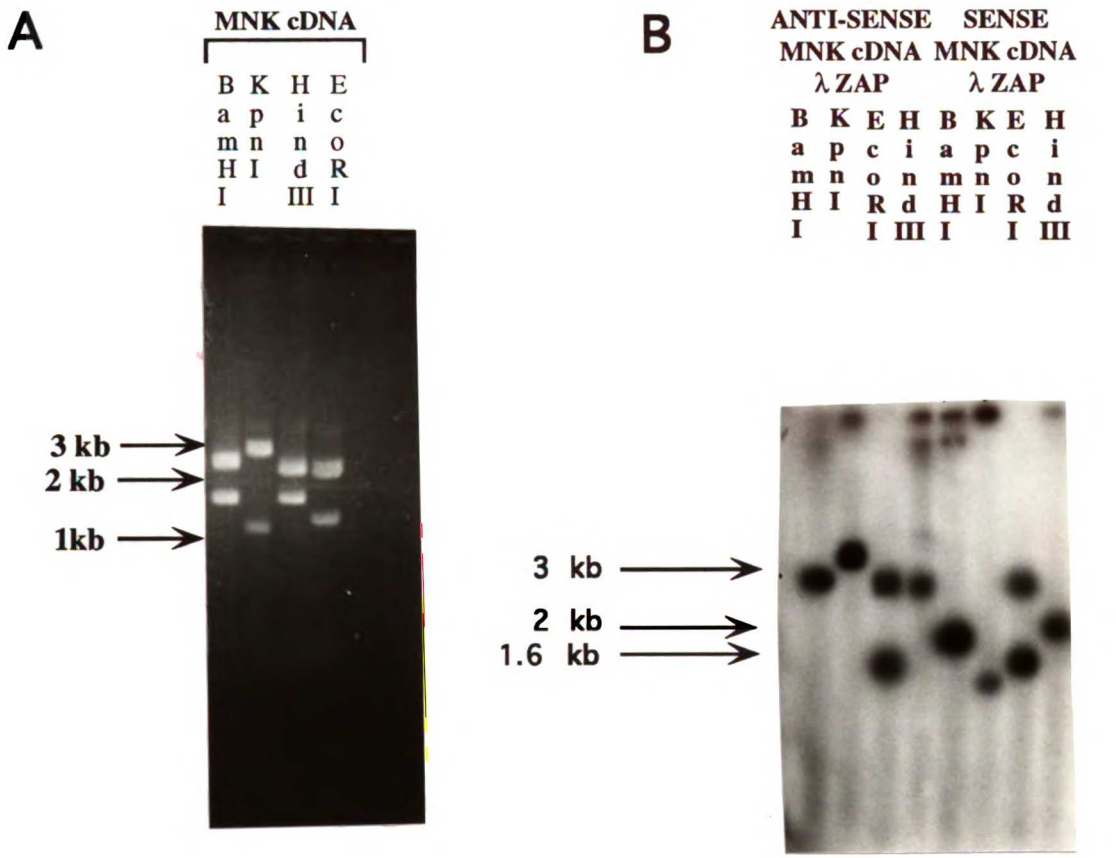


Figure18A and B: Restriction digest of λ ZAP clones indicate full length MNK ORF cDNA inserts. A:The inserts were PCR amplified from the λ ZAP clones containing the MNK cDNA inserts with primers to the 5' end and 3' end of cDNA. The long PCR protocol was used to amplify the 4.5 kb cDNA. [5 λ of phage eluate(1 plaque in 500 λ SM) was amplified with 1 pm 5' and 3' MNK cDNA primers in 50 λ reaction mixture 10 mM Tris-Cl pH8.8, .075 mg/ml BSA, 1.75 mM $MgCl_2$, 25 μM dNTPs 1λ of a mix of15 λ klentaq(Ab Pepide)/1 λ Pfu(Statagene) using the parameters D 95°C, 10 sec A 57°C, 30 sec, E 68°C, 7 min for 35 cycles] Restriction digestion of the product were run on a 1% agarose gel and visualized by EtBr staining. A representative clone containing the MNK cDNA is shown at left. The expected restriction fragment size are indicated in the previous figure. Appropriate size fragments were seen for all restriction sites.

B: The orientation of the MNK cDNA insert was determined by restriction digestion of λ ZAP DNA clones. In the figure to the right, examples of a sense and antisense λ ZAP clone DNAs were digested at 37°C overnight with appropriate restriction enzyme as indicated, run on .5% agarose gel at 50V for 5hrs, and alkali blotted. The blot was probed with a combination of four probes which span the MNK cDNA. A restriction pattern expected for a anti-sense insert were seen in the clone presented to the left, while a pattern expected for a sense insert were seen in the clone presented to right.

clone tested was amplified in one piece by a modified PCR technique (Barnes, 1994; Cheng et al., 1994) and individually hybridized with four control RT-PCR products which together span the complete ORF cDNA. Chemical cleavage analysis of these four hybridization products detected no mismatches in the cloned Menkes complete ORF cDNA (data not shown). It was concluded therefore that the cDNA clones likely faithfully represent the Menkes cDNA and proceeded to expression studies. The successful cloning of the complete cDNA in a λ vector in contrast to the unsuccessful attempts in plasmid vectors perhaps reflect that λ vector propagation results in *E.coli* cell death and any possible toxic effects of the cDNA are redundant.

Stable transfectants containing the Menkes cDNA

Stable human cell lines containing the complete cDNA in λZAP express™ vector were selected. The λZAP express™ vector contains a Neo^R marker as well as mammalian mRNA expression signals flanking the cloning site including a CMV promoter and a SV40 poly A site. As mentioned before, both sense and antisense constructs were obtained. Several different sense constructs and one anti-sense construct were transfected by electroporation of whole phage into the adenovirus transformed human kidney cell line A293 and individual Neo^R clones selected. A total of eight transfectants containing a sense clone and two transfectants containing a antisense clone were obtained and analyzed further. The A293 cell line was chosen because it reportedly gives good expression of constructs utilizing the λZAP express™ vector CMV promoter (Stratagene, pers. comm.). Southern analysis of the Neo^R clones demonstrated integration of the Neo gene into the genome of the cell lines (Figure 19). Additionally hybridization of genomic digests with probes to the Menkes cDNA identify extra hybridizing bands in addition to

the normal Menkes gene locus restriction fragment consistent with the integration of the Menkes cDNA (Figure 20).

Stable transfectants express epitope tagged protein

Stable transfected cell lines which express the epitope tagged Menkes protein were obtained. Individual Neo^R clones were analyzed by Western analysis for expression of the heterologous cDNA. Antibodies to the "Glu-Glu" epitope detected an approximately 200 kd protein in the A293 cell lines transfected with the sense construct, but not in Neo^R clones transfected with the antisense cDNA, or the control A293 cell line (Figure 21).

These cell lines should provide the reagents necessary for the immunolocalization of the Menkes protein, and the cDNA construct should allow complementation analysis. Preliminary experiments utilizing the α-Glu-Glu monoclonal antibody for in situ immunolocalization of the epitope tagged Menkes protein in the stably transfected cell lines was unable to provide definitive localization. Further work will be needed to determine the conditions for successful in-situ immunolocalization. Similarly, the preliminary attempt to obtain Menkes cell lines stably transfected with the complete ORF cDNA expression vector were unsuccessful. Further steps to be taken are described in the following chapter.

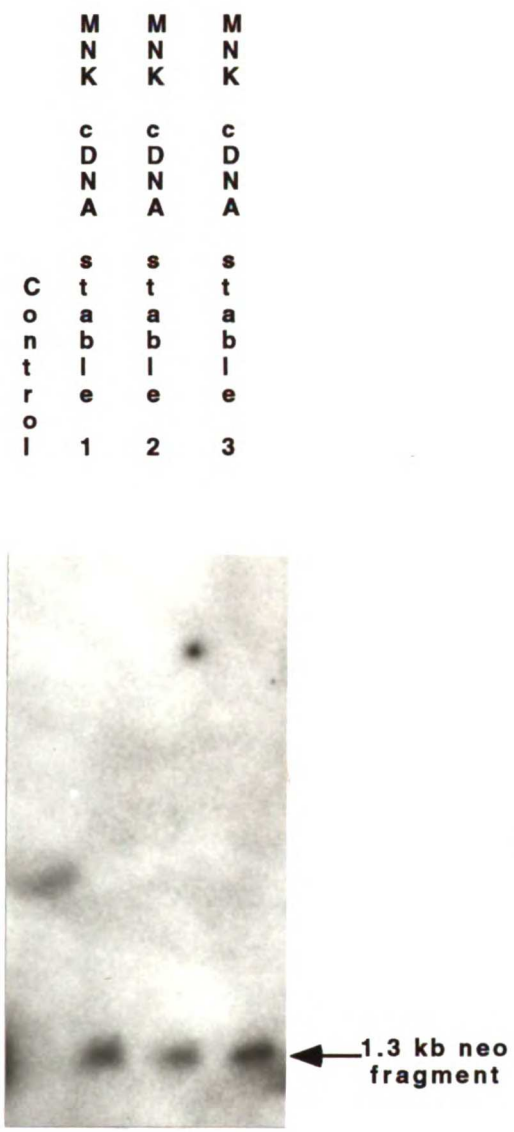


Figure 19: The Neo^R cell lines transfected with the Menkes cDNA in the λ ZAP express vector contain the Neo gene inserted into their genome. The control cell line is GM5659, a normal male cell line, the stable cell lines 1, 2 were Neo^R clones resulting from a transfection of the kidney epithelial cell line A293 with the Menkes cDNA in a sense orientation in the Stratagene λ ZAP express vector, while 3 contains an antisense construct. Transfections were with 5 X 10^6 purified whole phage into 1 X 10^6 A293 cells by electroporation with BRL Electroporator at 800 μFD, 350 V. Ten 100mM dishes were plated per transfection and Neo^R clones were selected with 400 μgs Geneticin(G418) for three weeks. Individual Neo^R clones were isolated and expanded. Genomic DNA was prepared from individual Neo^R cell lines and 10 μgs of each were digested with Bsu36I, which should release the Neo gene from the vector, and were run on a 1% TAE agarose gel, 50V for 16 hrs, and alkali blotted. The blot was hybridized with a Neo probe(Church hybridization buffer, 65°C, 15 hrs and washed in 2X SSC, .1% SDS at 65°C, 2X). The appropriate 1.3 kb Bsu36I fragment was detected in the three transfected cell lines and not in normal controls, indicating integration of the λ ZAP express vector cDNA constructs into the genome of the transfected cell lines.

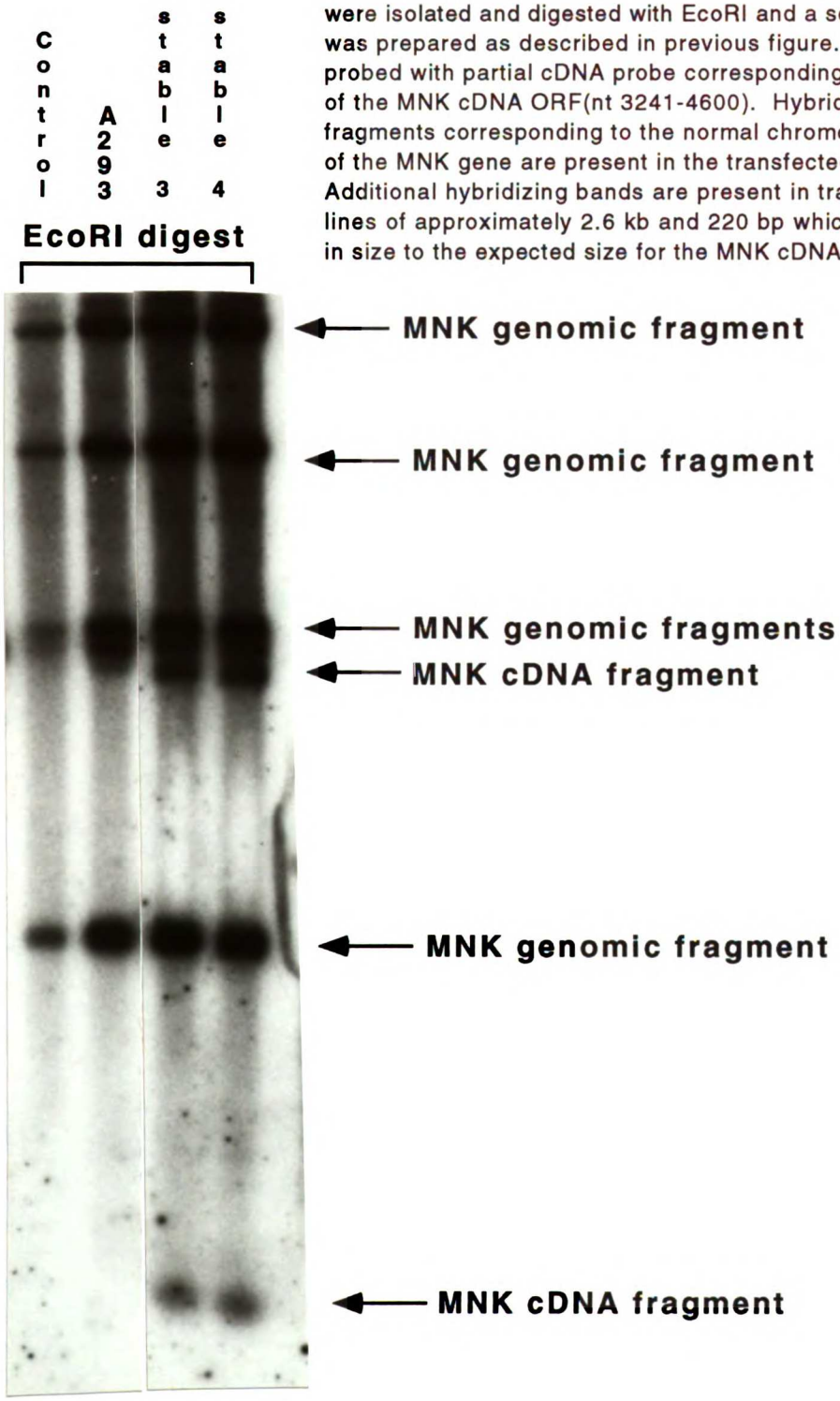


Figure 20: Menkes cDNA is present in genome of Neo^R clones transfected with λ ZAP express MNK cDNA constructs. Genomic DNA from stable cell lines were isolated and digested with EcoRI and a southern blot was prepared as described in previous figure. The blot was probed with partial cDNA probe corresponding to the 3' end of the MNK cDNA ORF(nt 3241-4600). Hybridizing fragments corresponding to the normal chromosomal copies of the MNK gene are present in the transfected cell lines. Additional hybridizing bands are present in transfected cell lines of approximately 2.6 kb and 220 bp which correspond in size to the expected size for the MNK cDNA.

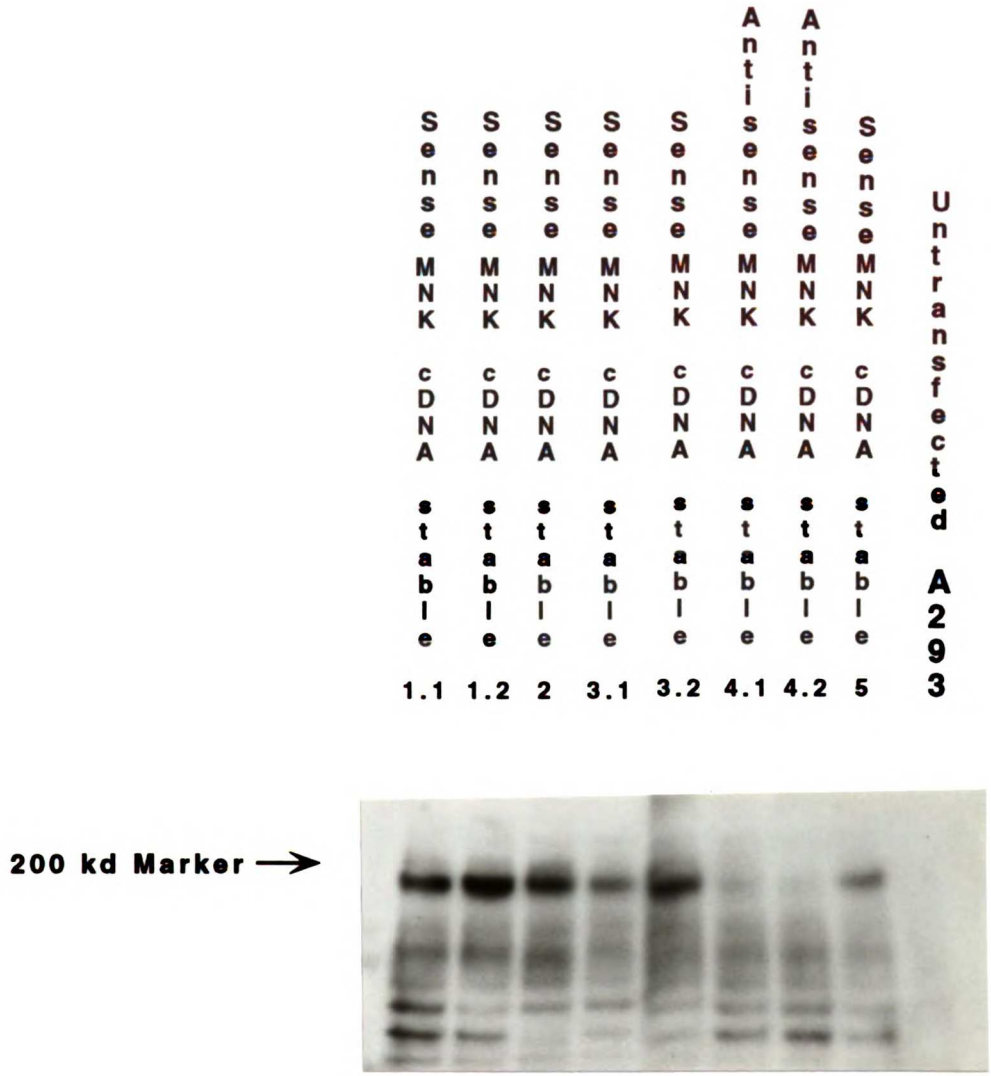


Figure 21: Antibody to "Glu-Glu" epitope detects a ~200 kd protein in cell lines transfected with sense MNK cDNA. Stable cell lines transfected with the λ ZAP express MNK cDNA constructs were analyzed for production of the epitope tagged Menkes protein by Western blot analysis with the anti-"Glu-Glu" antibody. A ~200 kd protein is present in the cell lines transfected with a sense construct but not in the antisense transfected lines or the untransfected A293 cell line. Protein preparations were prepared by lysis of approximately equivalent amounts of cells in 2X SDS lysis buffer(125 mM Tris-Cl pH6.8, 20 % glycerol, 4% SDS, 200 mM DTT) at 95°C for 5 min. Protein concentrations were determined by Biorad protein quantitation reagents and 50 µgs of total cellular protein were loaded per lane on a 4-15% SDS gradient gel(Biorad), run at 100 V for 1.5 hrs and semidry blotted onto supported nitrocellulose. The blot was blocked in 5% NFDM(Non-Fat Dry Milk) in TBST(100 mM NaCl, 10 mM Tris-HCl ph 8.0, .1% Tween-20) for 3 hrs. The mouse monoclonal primary anti-"Glu-Glu" antibody was added to a dilution of 1/3000 in TBST and incubated at 4°C overnight, rocking. The blot was washed four times in TBST, blocked 30 min in 5% NFDM in TBST, and incubated with a 1/2500 dilution of Donkey anti-mouse IgG-HRP(Amersham) for 1hr at 22°C, and washed four times in TBST. ECL detection reagents (Amersham) and autoradiography was used to visualize immunoreactive proteins.

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Chapter 6

Future Directions

Copper poses a dilemma for living systems. The capacity of copper to convert between oxidation states Cu(I) and Cu(II) accounts for its essential role in many proteins (Beinert, 1991). However, copper can also generate free radicals or oxidize cellular components through the same mechanism (Assseth and Norseth, 1986). The conflicting requirements of maintaining adequate copper for cellular functions while presenting toxic side effects necessitate close cellular regulation of copper levels (Harris, 1991).

Two inherited human disorders of copper metabolism highlight the dilemma between copper necessity and toxicity. Systemic copper deficiency and subsequent decreased activity of copper dependent enzymes underlie the clinical symptoms in Menkes disease (Menkes, 1988). In contrast, the clinical presentation of chronic liver cirrhosis and a progressive movement disorder in the autosomal recessive Wilson's disease results from copper toxicity (Danks, 1991). Copper accumulates in the liver and later in the course of the disease in other tissues including the brain and the eye. Defects in intracellular copper processing and transport underlie both the copper deficiency of Menkes disease and the copper excess and toxicity of Wilson's disease (Danks, 1989).

The identification of the Menkes disease gene and its homologue in the mouse, the Mottled gene, revealed a novel mechanism utilized by eukaryotic cells to transport copper via unique P-type ATPase with six N-terminal metal binding motifs (Chelly et al., 1993; Mercer et al., 1994; Mercer et al., 1993; Vulpe et al., 1993; Levinson et al., 1994). The recent identification of the Wilson disease gene (Bull et al., 1993; Tanzi et al., 1993; Yamaguchi et al., 1993)

and its rat homologue (Wu et al., 1994) and the demonstration that they encode close homologues of the Menkes disease P-type ATPase suggest mammals utilize these novel P-type ATPase for multiple copper transporting tasks (Bull and Cox, 1994). The Menkes and Wilson disease genes are expressed mainly in different tissues. The Wilson disease gene is expressed in the liver, kidney, brain and placenta while the Menkes disease gene is expressed in all tissues except the liver. It is possible that these proteins play analogous roles in different tissues, although the shared expression in some tissues suggest perhaps unique roles or redundancy in at least those tissues. Copper must be transported into multiple intracellular locations and it is not know whether the Menkes protein alone fulfills these multiple roles , or perhaps additional homologous organelle specific transporters could perform these transport tasks.

The homology of Menkes/Wilson copper transporting ATPase to the *E.hirae* CopA protein, a copper transporting ATPase in this bacterium, provided an example of remarkable conservation of form and function between bacteria and man (Odermatt et al., 1993; Solioz et al., 1994). Additional homologues in many different species have been isolated (see P-type ATPase section of Appendix), including two different homologues in *S.cerevisiae* (Beeler et al., 1994; Ramezani Rad et al., 1994). Two distinct homologues, excluding redundancy, implies distinct tasks for these proteins in the cells perhaps in the import or export of copper from cellular organelles. The transport systems for many other metals, both essential such as zinc and cobalt, and non-essential, such as cadmium and mercury, have not been elucidated and the cell may well utilize transport proteins homologous to the Menkes protein.

Future work

Although the identification of the gene is an important first step, substantial work remains to be done to determine the role of the copper transporting ATPase encoded by the Menkes disease gene in mammalian copper transport. As described previously the intracellular location of the protein is not known, and this information is critical for an understanding of its function. In addition there is no direct evidence that the Menkes protein, or any of the homologues are copper transporting ATPases. Biochemical and genetic evidence support this assertion but demonstration of copper transporting activity is lacking.

Cellular location

I would propose the following steps to be taken to continue this work. The production of stably transfected cell lines producing the epitope tagged Menkes protein provides a useful reagent for immunolocalization of the protein. Unfortunately, due to time constraints, only a limited number of attempts at in situ localization using the α-Glu-Glu antibody were possible. I believe a systematic variation of fixation, blocking conditions, and primary antibody and secondary fluorescent antibody concentrations or other parameters would eventually result in a reproducible fluorescent signal in one or more cellular locations. Double labeling with an antibody specific to the tentative compartment and with the α-Glu-Glu antibody would be need to confirm its localization. Alternatively cell fractionation of the overexpressing cell lines could be used to localize the expression of the protein. Different membranous compartments could be enriched and tested for immunoreactivity by western blot analysis with the α-Glu-Glu antibody.

One concern of these immunolocalization studies with the epitope tagged protein is that heterologous overexpression of the Menkes cDNA could result in mislocalization of the protein and leading to a spurious cellular localization. Antibodies to the native protein will be necessary to rule out this possibility.

As discussed in the previous chapter, the antisera prepared to the N-terminus of the Menkes protein detects a protein or proteins of the appropriate size in normal cells which should express the protein and in mutant cell which should not. Some attempts were made to increase the specificity of the antisera and preliminary results with antiserum prepared by another laboratory indicated that more stringent washes of western blots could increase the specificity although and some cost to sensitivity. Variations on this approach could lead to specific and sensitive antiserum. Other possible avenues of approach could be tried. For example, two dimensional electrophoresis could resolve a cross-reacting protein of identical molecular weight from the Menkes protein and allow utilization of the antisera for some experiments. Cell lines from Menkes patients could be examined in this way for expression of the Menkes protein. Cell fractionation followed by two dimensional electrophoresis an western blotting could allow immunolocalization of the protein. Two dimensional electrophoresis clearly would not facilitate in situ immunolocalization. Other possible means to increase the specificity of the antisera would be to pre absorb the sera against extract from a cell which should not express the Menkes protein but does express the cross reacting material, such as C.G. translocation cell line or other known null mutants.

In vitro copper transport

Cell lines which over express the Menkes protein could also facilitate development of an in vitro copper transport assay for the Menkes protein, to directly assess the copper transport activity of the Menkes P-type ATPase. In vitro assays for ion transport have been developed for other P-type ATPase most notably for the sarcoplasmic reticulum(SR) Ca^{2+} transporter (Clarke et al., 1989). In this SR Ca^{2+} ATPase system , the SR Ca^{2+} in a SV40 expression vector is transfected into Cos 1 cells and after several days the cells are harvested. A crude microsomal fraction is prepared which contains high levels of the heterologously expressed SR Ca^{2+} ATPase. Ion transport by the SR Ca^{2+} ATPase across the microsomal membrane is monitored by radioactive Ca^{2+} uptake into the microsomes. This assay system has allowed detailed characterization of the sarcoplasmic reticulum Ca^{2+} ATPase including the identification of residues critical to ion binding and specificity (Clarke et al., 1990; Clarke et al., 1989). An analogous system could be used to characterize the Menkes candidate gene product.

Microsome preparations from the stably transfected cell lines overexpressing the Menkes protein could be used in an analogous assay of transport using radioactive copper as a tracer. It would be necessary to first confirm expression of the Menkes protein in the microsomal fraction by Western blotting of a microsomal fraction from the cell lines. Conditions and procedures developed for calcium transport would be a starting point for optimization of copper transport conditions. Protein expression levels of the sense constructs in each preparation could be determined by quantitative ELISA assay to normalize for variation in expression levels. In the copper transport assay, aliquots of the microsomal fractions would be mixed with

radioactive copper and samples taken at periodic time points, and filter binding assay utilized to determine the amount of copper transported into the microsomes. Copper within the microsomal fraction would be expected to remain bound the filter paper similar to calcium in the SR Ca^{2+} ATPase protocol. Microsomal fractions from antisense transfected cell lines would serve to determine the basal level of copper transport. Once copper transport conditions are established, the basic parameters of the reaction would be determined including the rate of transport at varying copper concentrations and requirements for ATP. Various inhibitors of P-type ATPases including vanadate, a transition state analog of ATP (Sachs and Munson, 1991), could be tested for their efficacy in preventing the action of the Menkes protein.

Menkes cell lines show a specific defect in copper transport and show no alterations in Zinc or Cadmium transport (Packman et al., 1983). The specificity could be due to the intrinsic affinity of the Menkes gene product or the presence of redundant systems for zinc and cadmium transport. The in vitro system will allow direct consideration of this question. The specificity of transport could be assessed by determining uptake into the microsomal fraction of other radioactive metals including silver, cadmium, and zinc. The relative affinity of these metals for Menkes protein could be determined by competition assays with one radioactive metal and one non-radioactive metal competitor. A selective transporter would be expected to show a marked preference for copper transport whereas a non-selective transporter would not.

Once the basic parameters of transport are established, site directed mutagenesis of the complete open reading frame cDNA and establishment of

cell lines expressing the mutant variants will allow structure-function analysis. For example, the in vitro assay system could allow determination of the residues critical to ion binding. Cysteine, histidine, and methionine residues are residues involved in copper binding in other proteins so they may play a role in copper binding in the Menkes protein. I hypothesize two regions with potential metal binding residues, the N-terminus and the fourth transmembrane domain, may play critical roles in ion binding.

The N terminus contains six repeats of a twenty three amino acid putative metal binding motif each of which contains a conserved pair of cysteines and one conserved methionine. {See figures 4, 6 and 7 of Chapter 3.} Sequential truncations of the N terminus creating protein with fewer metal binding motifs could provide insight into their role in ion binding and transport. Similarly, concurrent replacement of both cysteines and the methionine in each motif with non-charged amino acids could allow inactivation of each individual motif while leaving the others intact and help determine if one or more motifs are critical for function. The rate of copper transport for these mutants at different copper concentrations would be examined closely since these mutations may not completely disrupt transport. Such an analysis has been used for the SR Ca^{2+} ATPase to identify mutations which result in subtle changes in Ca^{2+} transport. Copper transport of each mutant would be normalized to the protein expression of each mutant in the microsomal fraction to differentiate between mutants which grossly effect protein folding or expression from ones with selective effects on copper transport.

The fourth transmembrane domain of the Menkes protein contains three cysteine residues which may be involved in ion binding {See figures 4, 6 and 7

of Chapter 3}. Two of the cysteine residues flank a conserved proline residue while the third residue lies three residues toward the N terminus from the CPC motif. In most cation transporters, a hydrophobic residue such as valine or isoleucine immediately precedes the proline, while a glutamic acid or a valine follows the proline (Fagan and Saier, 1994). However, in Menkes and the other known metal transporters there is a conserved CPC motif which may play a role in metal ion transport (Bull and Cox, 1994; Silver et al., 1993). The residue following the proline has been shown to be critical for ion binding in the Ca^{++}-transporting ATPase of sarcoplasmic reticulum (Clarke et al., 1990). Site directed mutants could be generated containing replacements of one, two or three of these cysteine residues and assayed in vitro as described previously.

A complete cDNA also allows a test of ability of the gene to complement the Menkes disease cellular defect in copper export. Correction of the cellular phenotype would confirm its role in Menkes disease. Stable transfection is facilitated by a transformed cell lines expressing the mutant phenotype. Lymphoblast cell lines exist for multiple patients with Menkes disease and I have established several independent SV40 transformed cell lines from two Menkes patients which could be utilized. Both lymphoblasts and transformed fibroblasts represent an appropriate tissue for complementation analysis as they express the cellular phenotype of copper accumulation.

Two assays for complementation of the defect in copper export could be utilized. The first assay, a copper accumulation assay, is currently used in our and other laboratories for the biochemical diagnosis of Menkes disease (Horn, 1981). Cultured cells are grown in standard media which contains trace

amounts of copper for several passages. We assess copper content of the cultured cells by atomic absorption spectrophotometry of cell lysates normalized to cellular protein concentration. Cell lines from Menkes patients show significantly increased copper concentrations as compared to normal controls. The second assay quantitates copper export from cultured cells utilizing radioactive copper (Horn, 1981). Cultured cells are loaded with radioactive copper(Cu^{64} or Cu^{67}) for several hours and then transferred to copper free media. At periodic time points, cells are harvested and the radioactive copper remaining in the loaded cells is assayed. Cell lines from Menkes patients show similar initial rates of copper export but decreased rate of export at later timepoints.

Each stably transfected cell line could be analyzed with these two assays. The expectation if the Menkes candidate gene can complement the cellular phenotype would be normal copper levels and copper export in Menkes cell lines transfected with the sense construct. A continued abnormal phenotype would be expected in Menkes cell lines transfected with the antisense constructs. Clearly some variation in copper parameters could occur due to chromosomal position effects on expression of the constructs and therefore several stable transfectants will be tested for each construct. The normal cell lines with sense and antisense constructs represent controls to determine if ectopic expression of this gene at potentially non-physiologic levels leads to disturbances of copper homeostasis

The patients with Menkes disease were the inspiration for this work, are ultimately the beneficiaries and their concerns must remain paramount in future research on this disorder. A better understanding of the molecular

mechanisms of copper transport, including the particular defect in Menkes disease, will hopefully result in rationale treatment strategies for the affected individuals. The mouse model, Mottled provides an excellent system in which to test those treatments. The identification of the gene has already led to prenatal diagnostics for some families with a history of Menkes disease, and with continued support will be available in the future. I look forward to the day when an effective treatment or prevention is available to all who need it.

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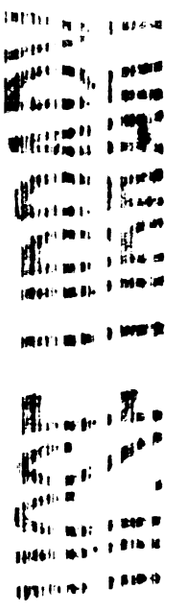

Appendix

Cellular copper transport

CELLULAR COPPER TRANSPORT

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INTRODUCTION

All living organisms, from bacteria to man, require copper, element 29, for continued growth and development and must obtain it from their diet. The nutrional requirement stems from the essential role of copper in the function of the numerous cuproproteins. However, copper in excess of cellular needs mediates free radical production and direct oxidation of cellular components with, detrimental effects. A critical balance must therefore be maintained by specialized cellular transport mechanisms to regulate intracellular copper content.

Copper transport in mammalian cells can be divided into three discernible but interrelated steps: copper uptake, intracellular copper distribution and utilization, and copper export. Understanding of each process requires definition of the phenomenon, measurement of the step in isolation, and the identification of the responsible components. Although proteins in the transport and utilization of copper have in some instances been identified, our current understanding of the cellular processes is limited.

There are two well defined copper uptake pathways used by mammalian cells. Copper bound to ceruloplasmin, and copper not bound to ceruloplasmin, enter the cell along initially distinct but ultimately convergent paths. In contrast to uptake of other cations, such as transferrin bound iron, no ligand accompanies copper into the cell. The protein components responsible for these entry steps, save for putative ceruloplasmin receptors, remain unidentified.

The processes controlling the distribution of copper to different cellular compartments and its delivery to copper proteins are poorly understood. Metallothionein may have a more limited role than originally suspected. and, other intracellular copper binding proteins, or glutathione, may instead

function in copper distribution. Inherited disorders with possible defects in intracellular copper transport, such as Indian childhood cirrhosis in humans or toxic milk in the mouse, should ultimately lead to an improved understanding of the mechanisms involved.

The human inherited disorders, Menkes disease and Wilson disease, spurred interest in and contributed significantly to our understanding of copper export. Copper accumulation in the liver of patients with Wilson disease, and in cultured cells of every other cell type in Menkes disease, results from defective copper export.We have only recently learned that the two diseases result from defects in homologous genes with similar function(s), expressed in different tissues. Both genes code for copper-transporting P-type ATPases, a class of integral membrane proteins which transport cations across cellular membranes in organisms as diverse as bacteria and man. Long suspected animal models of these diseases, the mottled mouse (Menkes disease) and the LEC rat (Wilson disease), were confirmed as animal homologues by molecular cloning studies. Insights into the mechanism of copper export from cells will result from the continuing characterization of these proteins.

Copper transport is required by all organisms. Study of copper transport in bacteria and yeast has identified proteins involved in aspects of copper transport, such as copper uptake, for which no mammalian proteins have been identified. These unicellular systems therefore provide a broad perspective on copper transport, suggesting experimental approaches to be taken in mammalian cells, and identifying proteins with potential mammalian homologues. In this review, we will first consider selected aspects of copper transport by bacteria and yeast. Mammalian cell copper uptake, intracellular copper distribution and copper export will then each be

discussed in individual sections. Mammalian metal responsive transcriptional regulation is not discussed in this review and the reader is referred to reviews on this topic[129, 195].. The reader is also referred to other reviews for details of organismal copper transport [62, 98].. We feel that a challenge in this field is to proceed from identification and delineation of transport steps, and towards the actual identification of the proteins and mechanisms responsible for the uptake, distribution, utilization, and export of trace metals by mammalian cells.

COPPER TRANSPORT IN UNICELLULAR ORGANISMS

An understanding of copper metabolism in unicellular organisms provides a foundation for the understanding of mammalian copper metabolism. Copper presents the same dilemma to bacteria, yeast, and mammalian cells: it is a vital yet deadly element. The complex multi-component copper handling systems identified in bacteria and yeast suggest the existence of a similarly complex system in the mammalian cell. Functional units in bacteria and yeast are likely to have functional if not structural homologues in mammals, and representative systems will be discussed (cf. Figure 1, Figure 2 and the section on P-type ATPases).

Bacterial Transport:Pseudomonas Syringae

Resistance of*Pseudomonas syringae var. tomato to* copper salts sprayed on crops for pest management led to the identification of the *cop* operon[20].. The blue color of*Pseudomonas syringae* colonies grown on copper reflects copper sequestration in the periplasm by proteins encoded by the *cop* operon, *copABCDRS*[25].. In one model based on mutational analysis and direct

biochemical studies and localization(figure 1), CopB transports copper across the outer cell membrane into the periplasm. Periplasmic CopA binds eleven copper atoms to multiple copper binding sites. CopA and CopB contain several novel eight amino acid motifs, X-Asp-His-X-X-Met-X-X-Met, which may bind copper[20].. A multicopper oxidase signature domain (such as that in ceruloplasmin) is also present in CopA, although biochemical confirmation of an enzymatic function is lacking [26].. Periplasmic CopC and inner cell membrane CopD cooperate in copper uptake into the cell from the periplasm[26]..

Bacterial Transport: E. Coli

Both plasmid and chromosomal genes govern copper homeostasis in *E.coli.* Six chromosomal genes or complementation groups, *cutA-F,* are likely involved incellular distribution [145]., while the plasmid encoded system, *pcoABCDRS,* may constitute a copper resistance system utilized in conditions of copper stress [144].. The predicted Pco proteins are structurally similar to the predicted Cop proteins from *P.syringae* [13].but the copper resistance mechanisms differ. In high copper,*P. syringae* accumulates copper in the periplasm. In contrast, *E.coli,* containing the *pco* operon plasmid, actually accumulates less copper than non-resistant *E.coli* [145].; the *pco* genes either prevent the entry of copper or increase export.

Genetic analyses of conditional copper sensitive *E.coli* mutants, and their copper import and export phenotypes, suggested a model for the role of each protein (figure 1)[13, 145]. CutA and CutB may be involved in copper import: the *cutA* mutant increases the maximum rate of copper import while the *cutB* mutant requires extra copper for growth and accumulates less copper than wild type. A three gene operon encoding two inner membrane proteins

and cytoplasmic protein of unknown function complements the cutA mutant [105]. *CutC* and *cutD* mutants show increased copper accumulation and may be involved in energy dependent copper export. CutE and CutF may deliver copper to copper proteins and to the export system. The recently cloned *cutE* gene predicts a 512 amino acid cytoplasmic protein with a single X-His-X-X-Met-X-X-Met motif similar to the motifs in CopA and CopB from *P. syringae.*[143].. Recent characterization of protein components such as CutA1-3 and CutE identifies novel proteins involved coincidentally in areas of copper transport for which there are as yet no known mammalian counterparts [cf. below].

Yeast copper transport

Copper uptake in *Saccharomyces cerevisiae* appears to be a carrier-mediated energy-dependent process[97].. Transport is independent of yeast metallothionein, Cup1, and relatively selective for copper (zinc or nickel only partially inhibit copper uptake). Copper may enter the cell as ionic Cu(I) ,and transits to intracellular proteins or compartments[96, 97]..

Unexpected insight into copper transport came from the study of iron transport in *S. cerevisiae* [22].. Three recently identified genes, *CTR1, FRE1* and *MAC1,* may play dual roles in copper and iron homeostasis in *S. cerevisiae. CTR*1 was identified in a screen for mutants with decreased iron uptake[31].. Surprisingly, increased exogenous copper restored iron transport of *ctr1* mutants. Ctr1 defects were shown to result in primary decreased copper uptake, with a secondary effect in iron transport probably due to reduced activity of Fet3, a multicopper oxidase required for iron uptake[6].. The *CTR1* gene has no significant overall homology to any known gene. Ctr1 is a plasma membrane protein, with possible metal binding motifs, including

three repeats of a nineteen amino acid motif containing four methionines each, and eleven double methionine motifs some of which are contained within the extended motif. The second motif , Met-X-X-Met, is similar to the proposed bacterial copper binding motifs in the (periplasmic) CopA and CopB proteins from *P. syringae* , and in the PcoA and CutE proteins of *E. coli* . Thus, the functional and sequence data suggest that Ctr1 is a novel-copper transport protein.

Two additional proteins involved in iron uptake may be involved in the uptake of copper. Fre1, an extracellular-facing iron reductase required for iron uptake[30]., is also a copper reductase and probably involved in copper uptake[74].. Transcription of the *FRE1* gene is negatively regulated by increased levels of both copper and iron [30, 74]., and copper ions also decrease the iron reductase activity of Fre1, suggesting a direct competition for Fre1 by the cations[92].. A newly identified regulatory protein, Mac1[74]. may modulate this transcriptional control of *FRE1* in response to copper. Mac1 may therefore regulate copper uptake via regulation of the expression of the copper reductase *FRE1*.

The study of the intracellular fate of copper in *S. cerevisiae* has focused on metallothionein. The insights into copper homeostasis gained by study of yeast copper metallothionein, Cup1, and its copper specific induction by the Ace1 protein, have been summarized [194].. The *CUP1* locus is not essential for yeast viability but is necessary for growth in the presence of elevated copper[60]., suggesting a detoxification function for the Cup1 protein, rather than a role in copper delivery to copper proteins. The cellular components responsible for the intracellular routing of copper are unknown, but these roles could possibly be filled by glutathione and phytochelatins[82]. The demonstrated copper-, cadmium- and zinc- induced synthesis of

phytochelatins in *S. cerevisiae* is consistent with a role in copper homeostasis although their function, especially when metallothionein is present, remains to be defined[192]..

Fortuitous insight into organelle copper transport and possibly copper export came from the study of *S. cerevisiae* mutants with defective vacuoles, the yeast equivalent of the lysosome[42].. Yeast with defects in the *VMA3* gene, which encodes a subunit of the vacuolar H^+-ATPase, are hypersensitive to copper, suggesting that the vacuole normally is involved in copper detoxification or export. This observation suggests the need to address the effect of other vacuolar mutants on copper sensitivity .

Conclusions

Copper transport in bacteria and *S. cerevisiae* highlights the complexity of copper metabolism and suggests avenues for future work in mammalian systems. Several novel potential copper binding motifs are shared by copper binding proteins. A methionine rich motif, X-His-X-X-Met-X-X-Met, was identified in the P. *syringae* CopA and CopB proteins, the E.coli PcoA and CutE proteins, and *S. cerevisiae* Ctr1. The conservation of this motif makes it likely that mammalian cells also take advantage of its copper binding properties. A key feature of bacterial and *S. cerevisiae* copper transport is the cooperation of several proteins. The CutA and CutB proteins both play a role in *E.coli* copper import, while the pairs of proteins CopC and CopD or CutC and CutD cooperate in bacterial copper export. Both Ctr1 and Fre1 are required for copper uptake in *S. cerevisiae* , with Fre1 performing the copper reduction necessary for uptake. Bacteria, *S. cerevisiae* , and mammalian cells all utilize the sequestration of copper to handle excessive copper: *P. syringae* retains copper in the periplasm and eukaryotic metallothioneins bind excess

copper. The *vma3* mutant points to a role for vacuoles or lysosomes in copper metabolism, possibly storage or detoxification. Structural and functional homologies between unicellular organisms and mammals are frequently found, as described below for the Menkes and Wilson disease genes, and it is prudent to consider bacteria and yeast for clues which may help us to understand mammalian copper transport.

MAMMALIAN COPPER TRANSPORT: CELLULAR UPTAKE

The mammalian cell utilizes at least two pathways for copper uptake. In one, the cell imports copper from the plasma copper protein, ceruloplasmin. Copper bound to albumin or histidine, or unbound copper, enters by a second route, herein designated the "free" copper pathway . Both processes are mediated by energy-independent facilitated transport, result in the entry of copper but not the extracellular ligand, and contribute copper to cuproproteins within the cell[18, 102].. The identification of the proteins involved and the elucidation of the relationship between the two processes are challenges for ongoing research.

Ceruloplasmin

Ceruloplasmin mediated copper uptake represents a novel cellular process for micronutrient transport [147].. Ceruloplasmin is the most abundant copper protein in plasma and contains 70-95% of plasma copper [62]. Ceruloplasmin has a ferroxidase activity with uncertain physiologic significance[147]., but clearly has a role in copper transport. Each ceruloplasmin protein tightly binds six to seven copper atoms to a variety of

copper binding sites[116, 147]., and can contribute copper to cells [18, 67, 101].and to intracellular copper proteins[29]..

Copper derived from ceruloplasmin enters the cell but the protein does not[132]., in contrast with, e.g., the co-uptake of protein and metal in transferrin mediated iron uptake. Sulfhydryl modifying reagents inhibit the uptake of copper, and implicate sulfur amino acids in the transport process. Inhibition of copper uptake by cuprous chelators and the stimulation of copper uptake by ascorbate suggest that copper uptake is as Cu(I) rather than Cu(II) [61, 131]...

A ceruloplasmin receptor may facilitate copper uptake by the mammalian cell. Specific ceruloplasmin binding sites on several cell types and tissues support the notion of a ceruloplasmin receptor [8, 56, 80, 81, 121, 163].. Potential receptors purified or enriched by a variety of affinity based methods differ in their size and composition[8, 119, 161].and there is no compelling evidence supporting one putative receptor over the others. The numerous proteins identified as putative receptors may represent components of this complex, all with some affinity for ceruloplasmin.

At least half of the copper from the ceruloplasmin first enters a membrane enclosed space, perhaps endosomes, which co-purifies with clathrin coated vesicles[39].. Radioactive copper can be tracked from this compartment later appears in copper proteins such as SOD1[39]. Understanding of ceruloplasmin mediated copper transport has evolved from uncertainty of its copper transport role to the characterization of potential ceruloplasmin receptors and a defined pathway. Such thorough biochemical characterization of ceruloplasmin mediated copper uptake sets the groundwork for the future identification of the components responsible for this process..

Free Copper Uptake: Intestinal Mucosa Apical Transport

Free copper not bound to ceruloplasmin follows a different path into the cell. Uptake by three extensively studied cell types, intestinal mucosal cells, fibroblasts, and hepatic cells will be considered separately. Although there are difference in the focus of studies and specifics of transport in each system, sufficient similarities support a unified view of copper uptake utilizing mechanisms common to all cell types.

Mammalian intestinal copper transport, both in studies of combined apical , intracellular, and basolateral copper transport and in studies which measure isolated apical copper transport from the intestinal lumen into the intestinal mucosa, show a saturable, presumably carrier-mediated, process[27, 175],. with at least one saturable transport component[175].. Intestinal luminal contents, such as amino acids [182]., ascorbic acid [177]., fiber and EDTA [174].affect combined intestinal copper uptake in varied ways. Studies which focus on isolated apical transport provide a clearer view of the process, and demonstrate copper uptake kinetics consistent with a mediated process at physiologic concentrations [12, 183],. with possible exceptions [174]. Both zinc [47]. and cadmium [40].inhibit combined intetinal copper uptake and one component of this inhibition may be via a direct block of apical copper transport. High luminal zinc concentrations result in immediate inhibition of copper transport [118]. One component of zinc inhibition of intestinal copper uptake may therefore be by direct interference with an apical copper transporter.

Free Copper Uptake: Hepatocytes and Fibroblasts

Although there may be some differences in the copper uptake of cultured hepatic cells and fibroblasts, there are several features strikingly shared across

species and cell types. As in ceruloplasmin mediated transport , extracellular ligands do not enter the cell with copper [43, 176]. Most studies support an energy independent, saturable, presumably carrier-mediated process [43, 104, 154, 164, 185],..with some exceptions[106].. Inhibition of uptake by other metals such as zinc or cadmium suggests the existence of a general metal ion carrier rather than a copper-specific carrier[43, 154].. Studies which have examined intracellular distribution of transported copper immediately after transport find significant amounts of copper in both cytoplasm and membrane bound fractions in both hepatocytes and fibroblasts[154, 180].

Histidine and albumin, have different effects on copper uptake. Histidine-bound copper comprises a significant fraction of circulating copper while albumin is a major copper binding protein in the portal circulation[62, 186].. Histidine at ratios equal to or less than 1:1 with copper do not effect copper transport. At higher concentrations, histidine acts as a weak competitive inhibitor of transport in hepatocytes [35].and as a noncompetitive inhibitor in fibroblasts[180].. Histidine facilitates copper uptake when albumin is present, and has been proposed as a physiologic carrier of copper in plasma for both hepatic and non-hepatic cells[35, 104]..

Copper initially bound to albumin may enter cells more slowly than unbound copper or copper bound to other ligands[35, 43, 176, 181].. However overall uptake of copper by hepatocytes in the presence of albumin may be higher than that of other cell types, possibly related to an additional hepatocyte intracellular copper binding protein[181].. Albumin binds copper predominantly via an N-terminal histidine rich region[138].and this site may be critical for facilitation of uptake, as copper bound to this site enters the cell more quickly than copper bound elsewhere to albumin[105].. Analbuminemia in humans [184].and animals [166, 178]. without defects in

copper uptake argues against an essential role for albumin in copper uptake. The physiologic role of albumin in the mediation of copper transport therefore remains controversial and requires further study.

Conclusions

A composite view of free copper transport into the cell emerges from the study of the different cell types. All are carrier mediated and not dependent on cellular energy. Zinc, or other metals, likely compete directly with copper for uptake. Extracellular ligands have variable effects which may reflect experimental or cell type specific differences. The similar parameters of non-ceruloplasmin copper uptake in the different cell types suggest that a single uptake process and common transport proteins are used by all cell types.

Copper routed through the two uptake systems, ceruloplasmin mediated and free copper uptake, at some point converges into a common path. It is likely , although not formally shown, that most non-hepatic cells posses both pathways. In vivo and in vitro studies demonstrated that albumin, histidine or ceruloplasmin is competent to deliver copper to copper proteins such as SOD1 in many of the same cell types[29, 55].. Several intriguing studies demonstrate an interaction between the two pathways perhaps at the copper entry phase. In one, free copper inhibited uptake of copper from ceruloplasmin[121].. The converse, inhibition of free copper uptake in the presence of ceruloplasmin, was noted in a another study on placental copper transport [107].. A further study found similar rates of copper uptake of free copper and of copper bound to ceruloplasmin [102].. The elucidation of the relationship between the two will likely provide insight into the individual mechanisms.

We present a unified model of copper uptake in Figure 3. In this model, a ceruloplasmin receptor brings ceruloplasmin to the cell surface where copper is released by reduction from ceruloplasmin. The copper is then captured by the , as of yet, unidentified passive copper carrier protein. Endocytosis brings the copper initially into the cell within endosomes from which it then exits into the cytoplasm via the copper carrier. Free copper or copper bound to other ligands utilizes the same reductase and follows the same path as copper from ceruloplasmin. The interface of these uptake systems with subsequent steps of intracellular copper transport remains unexplored and are the next frontier for investigation.

INTRACELLULAR COPPER DISTRIBUTION

Copper and copper proteins are distributed throughout the cell and in all cellular organelles including the nucleus, mitochondria, lysosomes, endoplasmic reticulum and cytosol[99]. One-quarter to one-half of cellular copper is cytosolic, with slightly less in the nucleus, and a smaller but significant amount in the mitochondria and endoplasmic reticulum. Copper proteins are similarly located in all cellular compartments. Examples include superoxide dismutase 1 in the cytosol and (perhaps) peroxisomes [28]., cytochrome c oxidase in mitochondria [84]., lysyl oxidase in the golgi and secretory organelles[86]., and metallothionein in the cytosol, nucleus and lysosomes [70, 71].

The means by which the cell delivers copper to cuproproteins, regulates intracellular copper levels, and compartmentalizes copper are poorly understood. Metallothioneins (MTs) had been proposed for multiple roles in copper distribution, regulation and delivery. Recent work questions a central role for metallothionein (MT) in copper distribution, and instead supports a

primary role in detoxification either directly through copper sequestration or through SOD-like activity of the copper bound MT[168].

Experimental and clinical investigations suggest that MTs do not play a role in delivery of copper to proteins responsible for copper export. Zinc, which directly inhibits intestinal apical uptake of copper (cf. above) also induces MT synthesis in intestinal mucosal cells after prolonged exposure[142, 151].. In intestinal mucosal cells of animals fed high zinc diets, copper uptake is reduced and more copper is bound to MT, but basolateral copper transfer across the serosal surface is unaffected [47, 48].. A clinical study similarly showed increased intestinal MT levels and decreased copper absorption after zinc treatment [193].. Copper has a higher affinity for MT than zinc[41].so it has been proposed that copper displaces zinc from MT [58]., trapping copper which enters the cell, and diverting that copper from normal basolateral export pathways. It follows that intracellular copper transit from the apical transporter to the basolateral transporter is unlikely to occur via MT under normal physiologic conditions.

The generation and breeding of mice simultaneously inactivated for MT-I and MT-II genes provide a direct test of the role of MT in copper metabolism[103, 113].. The double knock-out mice are liveborn, have no detected developmental abnormalities, and reproduce normally. Since inherited or environmental copper deficiency leads to multiple developmental defects, it must be concluded that either these MTs play no essential role in delivery of copper to cellular enzymes, or that redundant systems such as other MT isoforms [130, 137, 160]. can compensate. The knockout mice do show an increased sensitivity to cadmium toxicity, supporting a role for MT in protection from excess levels of at least one metal ion [103]..

[illegible]

Glutathione (GSH) rather than MT may mediate copper incorporation into nascent cytosolic apo-proteins requiring copper, and may also contribute to cellular defenses against copper toxicity. High levels of copper glutathione were thought to mediate copper resistance in copper selected hepatoma cell lines[50]., although reservations have been expressed [80]..Virtually 100% in vitro reconstitution of Cu-metallothionein and Cu-SOD from copper free apo-proteins and Cu(I)-GSH are consistent with a copper delivery role for glutathione[5, 24, 46]. In contrast, in vitro reconstitution studies of apo-SOD by Cu-MT found limited reactivation, dependent on non-physiologic oxidation of MT[52].. Although further work is required to confirm the in-vivo function of glutathione, glutathione depletion did prevent copper delivery to apo-SOD [159]. and apo-MT[158]..

Other cytosolic components such as the 38kd and 50kd copper binding proteins isolated from hepatic cytosols may contribute to the intracellular copper distribution[128].

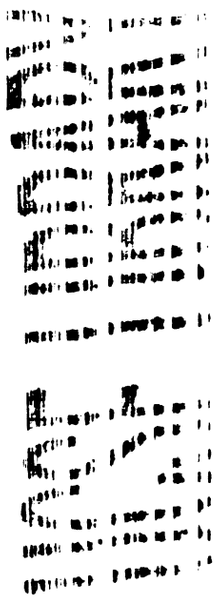
While transport of copper into membrane organelles is poorly understood, lysosomal transport is clearly a critical step in copper disposition. Hepatic copper overload leads to a disproportionate increase in lysosomal copper[88, 188]., possibly bound to MT. In mutant *beige* mice with abnormal lysosomes, hepatic lesions develop rapidly in response to a copper load [63].. We have already seen (above) that yeast with defective vacuoles (lysosome equivalents) are hypersensitive to copper [42].. The lysosome therefore may be a depot for excess copper and assist in the management of copper stress.

Other disorders of copper metabolism, such as the toxic milk mouse, may help us to identify critical components of cellular copper transport. Pups born to and nursed by toxic milk homozygotes exhibit curly whiskers, hypo-pigmentation, stunted growth, abnormal motor behavior and tremors, and

die as neonates[140].. Both heterozygous and homozygous pups born to homozygous mothers are affected, and the disease manifestations result from an autosomal recessive disorder of copper metabolism in the *mother*. Pups are born copper deficient and the milk of homozygous females is copper deficient. Copper treatment or cross fostering to a normal lactating female restores the copper status of the pups and they develop normally. The toxic milk females therefore manifest a defect of copper transfer into the placenta and into milk.

Male and female homozygous toxic milk adult mice also accumulate excessive copper in the liver, and have depressed ceruloplasmin levels[10]. As in Wilson disease, increased liver copper initially accumulates diffusely in the cytoplasm bound to MT[141]., with lysosomal accumulation in later stages of the disease[66]. However , the cytopathology of hepatic copper accumulation in toxic milk differs from Wilson disease[10, 66]., and suggests a different etiology. The role of MT in this disorder is controversial. In one study, increased MT mRNA expression was thought to be a secondary response [111]., while other workers found reduced MT mRNA expression but increased MT protein stability [85].. Further study is clearly warranted, not only to elucidate the basis of this disorder, but also to understand the tissue specificity of this disorder.

A combination of environmental and genetic factors may result in the disorder Indian childhood cirrhosis (ICC) [4, 34, 73].. Children with ICC accumulate massive amounts of copper in their liver, develop cirrhosis and liver failure, and, until recently, invariably died in infancy. The disorder can be treated with D-penicillamine[9].. Serum ceruloplasmin levels are normal [73].. The copper accumulates in the cytoplasm, possibly as copper-sulfur-



protein complexes[2, 9]. ICC livers exhibit a characteristic pathology distinct from other liver diseases, including Wilson disease[73]..

The use of copper cooking utensils in Indian households and the high copper levels in milk boiled in such vessels have led some to suggest an environmental etiology[120].. However, segregation analysis of 120 families with ICC suggested an autosomal recessive inheritance pattern[2].. In five non-Indian families with children with ICC, no source of increased copper intake was found[3, 65, 90, 100].. These included two different American families with multiple affected sibs, with consanguinity in one of the families [1]. Recent examination of ICC in patients in India found that in 46% of cases the use copper vessels in food preparation was denied, indicating that dietary copper was not the responsible agent[155]..

Biochemical abnormalities in cell lines from American patients with ICC strengthened the argument for a genetic etiology and provided the first molecular handle on ICC[57].. Cultured fibroblasts from patients with ICC, although demonstrating normal copper uptake and levels, had disturbed histology and cellular response to metal stress. Dilated rough endoplasmic reticulum, vesicular aggregates and fibrillar whorls (reminiscent of the liver pathology in ICC) were evident in the fibroblasts, suggesting a systemic rather than liver specific defect. Even more striking, basal and metal induced MT mRNA and MT protein synthesis were severely depressed . Induction of MT by copper, zinc and cadmium was defective, while MT induction by the glucocorticoid hormone was normal. MT protein turnover was normal and no mutations were detected in the hMTIIa coding region or promoter. This work suggests a genetic defect in MT synthesis in response to metals, perhaps in a metal-responsive metallo-regulatory protein. Although there is now strong evidence for a genetic etiology in at least some cases of ICC, this does

not rule out a potentiating role for dietary copper, or even the existense of two distinct disorders , one with a genetic origin and the other environmental etiology [65]..

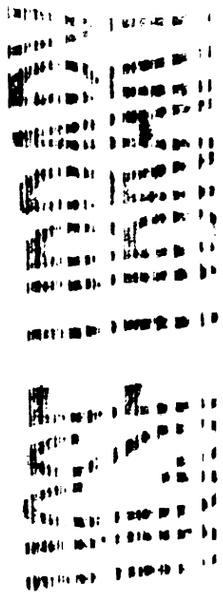
RELEASE OF COPPER FROM CELLS

Menkes Disease

Two human inherited disorders illustrate the importance of cell copper release. In autosomal recessive Wilson disease and the animal model , the LEC rat, decreased liver copper export results in copper-induced chronic liver disease and to pathologic changes in the brain, kidney and eye[33].. The features of Wilson disease reflect the effects of *copper toxicity.* In contrast, in X-linked Menkes disease and the animal model, the mottled mouse, defective copper export causes trapping of copper in some tissues (notably intestinal mucosa and kidney), leading to failure of copper delivery to other tissues. The clinical features of Menkes disease, therefore, result in large measure from a systemic *copper insufficiency*[33]..

Patients with Menkes disease manifest pili torti, hypopigmentation, growth failure, skeletal defects, arterial aneurysms, hypothermia, seizures, and progressive degeneration of the central nervous system, with death in early childhood [109].. Hemizygous male mottled mice can exihibit many of these same pathologic findings[68, 114].. Deficiencies in cuproenzymes in multiple tissues—e.g. tyrosinase (hypopigmentation), lysyl oxidase (defective collagen, elastin cross-linking), and dopamine-ß-hydroxylase (catecholamine production)—are likely responsible for some of the features [32, 135]..

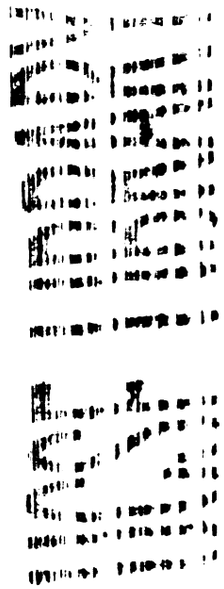
Defective copper export is the basic cellular defect in Menkes and mottled. With the exception of hepatocytes [36]. and cultured chorionic villus cells [170].,



all tested Menkes or mottled cultured cells exhibit excessive copper accumulation[33].. Menkes/mottled cells have a specific defect in copper efflux, with normal uptake, and with normal transport of cadmium and zinc in mutant cells under experimental conditions[64, 124, 125]. Increased concentrations of MT protein and mRNA have been demonstrated in Menkes/mottled cells [33, 83, 88a, 91, 122, 126] but the regulation of MT synthesis is normal [91, 122, 126]., suggesting that increased metallothionien is a secondary effect.

The Menkes and Mottled Genes

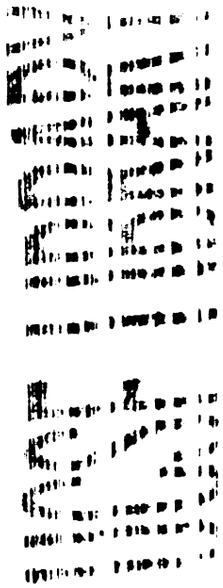
Three groups [23, 112, 179]. recently isolated the gene responsible for Menkes disease. The gene was identified by a positional cloning approach, based on gene localization to the Xq13 region of the human X chromosome by analysis of linkage[171]. and chromosomal rearrangements[79, 172, 173]. The gene is expressed in all tissues tested except liver, a pattern consistent with the Menkes phenotype. Sequence analysis of the MNK cDNA and the predicted 1500 amino-acid protein revealed that the Menkes locus likely encodes a copper-transporting ATPase of the P-type[179].. Diverse mutations in the MNK gene result in abnormal expression of the MNK gene [23, 37, 179].. Approximately 16% of patients with severe Menkes disease were found to have partial, non-overlapping deletions of the MNK gene [23, 179]. In patients without large gene deletions, independently occurring mutations in the *MNK* gene were detected, were different in each family, and were predicted to have a severe effect on the structure or expression of the Menkes protein [37].. From results to date, it appears that mutations unique to each family are responsible for the vast majority of cases of Menkes disease.



The *Mo* locus was mapped on the mouse X-chromosome near the phosphoglycerate kinase (*Pgk1*) locus [11]., a region homologous to the human Xq13 region containing the MNK gene. The mouse homologue of the MNK gene was cloned [94, 110]., was shown to map near Pgk1, and was observed to be expressed in the same tissues as MNK. In a severe mottled mutant (Mo^{dp}), there was no expression of the mouse gene in the hemizygote, but the exact molecular lesion in the *Mo* gene responsible for Mo^{dp} remains to be defined [53].. In a second, milder mutant (Mo^{blo}) , there was also abnormal expression of the gene [38, 94, 110].due to a splice site mutant in theMo^{blo} gene. These data suggest that the mouse homologue of the *MNK* gene is the *Mo* gene, and the mottled mouse is the murine model for Menkes disease.

Milder variants of Menkes disease and mottled may provide insights into the function and intracellular location of the Menkes/mottled copper transporting ATPase . One such variant is the occipital horn syndrome (OHS), a connective tissue disorder characterized by hyperelastic skin, bladder diverticulae, and skeletal abnormalities, including bony exotoses of the occiput[89].. The disease is sometimes accompanied by mild neurologic impairment, in contrast to the severe neurologic degeneration of Menkes disease.

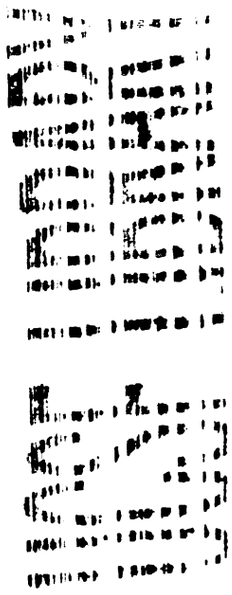
OHS is likely an allelic variant of Menkes disease. In both diseases, intestinal absorption of copper is deficient, serum copper and ceruloplasmin are low, cultured fibroblasts accumulate copper [87]., and a number of the connective tissue manifestations are similar in the two disorders. The Mo^{blo} mouse exhibits abnormalities reminiscent of those seen in OHS[114]. providing in at least one mouse mutant an example of a connective tissue phenotype similar to OHS. The clinical features can be related to a secondary deficiency of lysyl oxidase[17, 87]., which is encoded by a gene on human chromosome 5 [59].



A preliminary analysis revealed that *MNK* mRNA was greatly reduced in fibroblasts from two unrelated patients with OHS [93].. Two groups [38, 76]. detected mutations in OHS which affect the efficiency of normal splicing. Splice donor or acceptor site mutations resulted in the expression of not only an abnormal mRNA (due to exon skipping), but also some quantity of normally spliced message. Analysis of Mo^{blo}[38]. revealed a splice donor mutation which also results in both abnormal exon-deleted transcripts and mRNA of normal sequence. Both groups [38, 76].suggested that such subtle splicing mutations permit the synthesis of a small amount of normal copper transporting P-type ATPase protein in OHS and other mild variants.

The primarily connective tissue phenotype of OHS and $Mo^{blo,}$ in the face of some postulated amount of normally functioning Menkes/mottled transport protein, suggests that lysyl oxidase may be particularly sensitive to defects in the activity of the Menkes/mottled copper-transporting ATPase--- in contrast to, e.g., tyrosinase, cytochrome c oxidase or tyrosinase [69, 108, 123, 146].. It has been suggested [38]. that such sensitivity of lysyl oxidase---a secreted enzyme, in contrast to other cuproenzymes--- could reflect a requirement for the Menkes/mottled copper transporting ATPase in the transfer of copper to the specific cell compartment in which holo-lysyl oxidase is formed. Other cuproenzymes would be hypothesized to be located in cellular compartments more accessible to available copper, without the requirement of an additional transport step mediated by the Menkes/mottled proteins.

Copper efflux and cell fractionation studies are consistent with the notion that the copper accumulation of Menkes cells results from a defect in copper translocation across a *subcellular* compartment, rather than a plasma membrane defect in copper export [33, 64, 123, 127].. Other data suggest defective entry of copper into a number of cell compartments. Decreased copper content

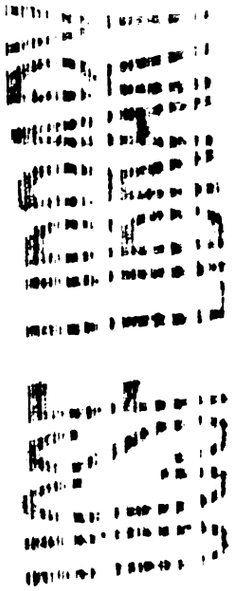


in mitochondria and lysosomes has been directly demonstrated in mutant cells by biochemical measurements[83]., electron microscopic cytochemistry [83]., and cell fractionation experiments [127].. The biochemical data and mutant phenotypes suggest that the Menkes/mottled disease gene products may be important for copper delivery to mitochondria and lysosomes, as well as essential for copper delivery to secretory path organelles such as the the endoplasmic reticulum or Golgi. Given the copper export defect in Menkes/mottled cells, it is reasonable to propose that normal pathway of copper export may include one of these intracelluar compartments. (Figure 3).

Wilson Disease

Wilson disease is an autosomal recessive disorder classically characterized by the coexistence of progressive neurologic findings, chronic liver disease with cirrhosis, renal tubular dysfunction, and pigmented corneal rings (Kayser-Fleischer rings). Neurologic symptoms may include behavior disturbances, dysarthria, and movement disorders. The age of onset ranges from 4 to 50 years,with an earlier age of onset generally associated with hepatic disease, often without neurologic manifestations . The copper content of liver, brain, kidney, and cornea is increased[162]..

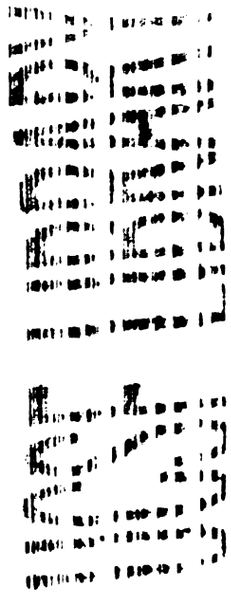
Impaired intracellular transport of copper results in decreased biliary excretion of copper and reduced incorporation of copper into ceruloplasmin. Ceruloplasmin levels and total serum copper concentrations are decreased in most patients, whether measured by enzymatic activity or immunochemical methods[98, 162].. Normally, copper appears to be incorporated into ceruloplasmin in the rough endoplasmic reticulum (RER) immediately following ceruloplasmin synthesis , in a step independent of ceruloplasmin secretion[54, 150].. Wilson disease leads to a perturbation in incorporation of



copper into ceruloplasmin and/or of ceruloplasmin secretion, perhaps because of defective copper transport into the organelles of the secretory pathway

In the early stages of Wilson disease, copper accumulates diffusely in the cytoplasm [4].bound to MT. Later in the disease copper is sequestered as copper-associated protein in lysosomes[4].. In contrast, in hepatic copper overload due to other causes (biliary atresia, nutritional overdose) copper first increases rapidly and disproportionately in the lysosome[4, 162].. It has therefore been proposed that the biliary secretion defect in Wilson disease may be related to the failure of transport of copper into lysosomes [51, 162].. It appears that both Menkes disease and Wilson disease can lead to defects of a secreted protein (lysyl oxidase and ceruloplasmin, respectively) , as well as to defects in copper transport into the lysosome. It is therefore possible that analogous transport steps in the RER, lysosomes and/or other organelles are mediated by homologous proteins in liver (Wilson disease) and other tissues (Menkes disease).

Studies of autosomal recessive copper toxicosis in the the Long Evans Cinnamon (LEC) rat revealed parallels to Wilson disease [115, 153]., including copper excess in liver and kidney, low serum ceruloplasm [95]., decreased biliary excretion [167]., and increased copper in certain sectors of brain [165].. There is early failure of transfer of copper from a cystosolic to a noncytosolic compartment in liver[189]., reminiscent of the anomolous cellular distribution in human Wilson disease. Finally, the hepatitis can be prevented by treatment with D-penicillamine, a copper-chelating agent used to treat Wilson disease[162].. A second proposed animal model, Bedlington terrier copper toxicosis [72].differs from Wilson disease in hepatic copper handling[162]., and maps to a probable different genetic locus[193a]..



Molecular Genetics of Wilson Disease

The gene for Wilson disease maps to the long arm of chromosome 13 [45].. The Wilson disease gene was identified by three groups of investigators [14, 15, 133, 169, 191],. based on positional cloning strategies, and on the hypothesis [179].that the Wilson disease protein might bear similarities to the Menkes disease P-type ATPase. Wilson disease (WND) cDNA expression is limited to liver, kidney and placenta, in contrast to the widespread expression of the Menkes gene. The WND gene encodes a 1411 amino acid P-type cation transporting ATPase [15, 169]., with 56% overall identity to the MNK gene product [14, 134]. The rat homologue of the Wilson disease gene has also been isolated, and deficient expression demonstrated in LEC rat liver due to a partial genomic deletion [149, 187, 190].

There appear to be no large gene deletions in Wilson disease patients, but rather a predominance of single base changes or small deletions, causing frameshifts and protein truncation [15, 133, 169].. Importantly, unrelated patients were found to be homozygous for an *identical* mutation[15].. In a related finding, two different disease mutations were observed in linkage disequilibrium with microsatellite marker haplotypes that represented common disease associated haplotypes in American and Russian populations[133, 169]. . A few mutations therefore appear to account for a significant fraction of cases in specific populations, and such epidemiologic correlates should find application in clinical molecular diagnosis. This pattern is different from that of Menkes disease [23, 38, 112, 179]., in which large deletions account for a significant fraction of mutations, and every family to date has had a *different* mutation.

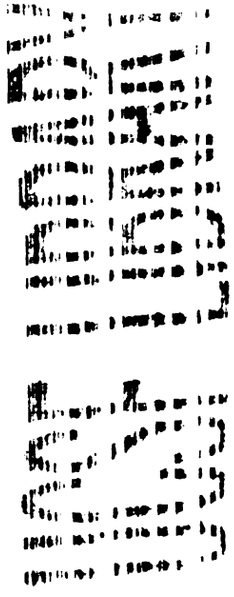
Analysis of WND mRNA revealed that the brain (and possibly kidney and placenta) contains transcripts with several different combinations of skipped

exons[134, 169]... One of these mRNAs would result in a truncated protein, and another would lack the transmembrane domain containing the CPC motif, thought to be essential for cation transport [134].. It has been speculated that such alternative splicing could be a regulatory mechanism, or result in a gene product with a different function from that of the liver protein[134].. These speculations are intriguing because of the involvement of other tissues in Wilson disease [162], and the disassociation of hepatic and neurologic symptomology in the LEC rat [115, 153, 165].and in some studies of the effects of liver transplant in humans[152]..

P-TYPE ATPases

The Menkes and Wilson disease genes and their respective animal homologoues encode copper transporting P-type ATPases[14, 15, 94, 110, 169,187]. P type ATPases transport cations using ATP. and form a family of integral membrane proteins in bacteria, fungi, plants and animals. This family of over seventy members includes Ca^{++}, $Cu^{+/++}$, Cd^{++}, H^+, K^+, Mg^{++}, H^+/K^+, and Na^+/K^+ transport ATPases[19].. All members are greater than 100kd, with between 6 and 10 transmembrane domains [7], and likely function as monomers to pump ions through a membrane. P-type ATPase form sub-families according to the ion transported. For instance, the human H^+ transporter is more closely related to other H^+ transporters rather than to a human Ca^{++} transporter[44]., and copper-transporting ATPases are more related to each other than to other P-type ATPases .The Menkes and Wilson disease gene products and their respective homologues bear the greatest similarity to the family of known and proposed *copper* transporting ATPases.

A model of the Menkes/Wilson copper transporting ATPases [15, 134, 179]. is presented in Figure 4. Copper is proposed to initially bind to the N-



terminus of the protein via six metal binding domains . Copper ions are then passed to the P-type ATPase core. Phosphorylation of an invariant aspartate by an ATP bound to the ATP binding/kinase domain, followed by dephosphorylation of the same residue by the phosphatase domain, leads to a conformational changes in the protein, putting probably one or two cations on the other side of the membrane. For some P-type ATPases, such as Na^+/K^+ ATPase, counter ions traverse in opposite directions, and unidentified counter ions may exist for copper.

Genetic and biochemical studies support a role for those related proteins in copper transport, although direct biochemical demonstration of copper transport is lacking. The *E.hirae* CopA and CopB proteins were the first described members and most extensively characterized[157]. Phenotypes of*E.hirae* individually deleted for the *copA* and *copB* genes support a role for the CopA protein in copper uptake and the copB protein in copper export[117].. Disruption of the *ctaA* gene in *Synecocchus 7942* results in increased copper tolerance and the CtaA protein is proposed to import copper[136].. In contrast, deletion of the *pacS* , an additional *Synecocchus 7942* ATPase, results in copper hypersensitivity. PacS is located in the membrane of the thykaloid(similar to a chloroplast) of the bacterium and was hypothesized to export copper from the organelle [77, 78]. .Additional copper transporting ATPases have been described in *S. cervisiae* [139],. *R. Mellioti* [75]., Helicobacter pylori, mycobacterium, and Listeria monocytogenes.

One striking feature of the copper, and other metal, transporting ATPases [156] are potential copper binding motifs in the N-terminus of the proteins. The N-termini diverge between families of P-type ATPase of different ion specificity. The N-terminus of most of the copper transporting ATPases are related to each other and all contain potential copper binding motifs. These

motifs in the copper transport ATPases are similar to a metal binding motif originally defined in mercury detoxification proteins and cadmium transporters, and consist of a highly conserved core of about 30 amino acids within an extended 72 amino acid motif[134].. A pair of cysteine residues in the motif, which has been shown to be critical for metal binding in a mercury protein[148]., likely plays a role in the copper binding. The Menkes/Mottled/Wilson P-type ATPase contains six of these motifs, the LEC gene - five, the yeast homolog, Ccc2 - two and the rest one. The domains in the copper transporting ATPase show greater similarity to each other than to the motifs in the mercury proteins and cadmium transporters, suggesting an intrinsic ion specificity[156].. The *E.hirae* CopB protein does not contain one of these motifs but instead contains an alternative metal binding motif homologous to the methionine rich motif in *P.syingae* CopA and CopB[117].

OVERVIEW AND CONCLUSIONS

Common themes in copper transport in unicellular and mammalian organisms discussed in this review reflect the requirements for copper in basic cell biochemical processes and the toxicity of copper excess. Among the diverse organisms, examples of protein components involved in copper transport, uptake, intracellular distribution, and export have been identified . Whether in bacteria, yeast or mammalian cells, copper import requires the coordinate function of several proteins with both metal binding and catalytic domains in a mediated transport step. In at least*S.cerevisiae* and mammalian cells, these components appear to function in the transport of additional metals, such as iron, or can be affected directly by other metals such as zinc. The potential reactive properties of copper require intracellular mechanisms

for copper delivery to cupro-proteins and for detoxification. Glutathione, rather than metallothionein may fill the cytosolic copper delivery function in mammals. Additional proteins play a role in bacteria and suggest that a more complex picture may emerge in mammalian cells. Cells across species deal with excess copper either by binding it to specifc detoxification proteins, such as mammalian or *S.cerevisiae* metallothioneins and *P.syringae* CopA; by transporting copper into an isolated cellular compartment, such as the lysosome, vacuole (*S.cerevisiae*), or periplasm (*P.syringae*) ; or by exporting it out of the cell. *S.cerevisiae* and bacterial copper transport proteins are likely to have functional homologues in mammalian sytems. Individual domains of these proteins, e.g. , metal binding domains, reductase domains, and ATPase domains are likely to have structural homologues among species. Exact function may differ depending on the needs of the different cell types and the extracelluar environment of the cell. A striking example is provided by the evolutionarily conserved copper transporting ATPases, such as the Menkes and Wilson disease gene products and the *E.hirae* CopA protein, which export copper from cells either directly or via intracellular compartments. We have attempted in this review to integrate the knowledge of copper transport in different organisms, to highlight the commonalities, underscore the gaps in knowledge, and hopefully provide paradigms for future research.

ACKNOWLEDGMENTS

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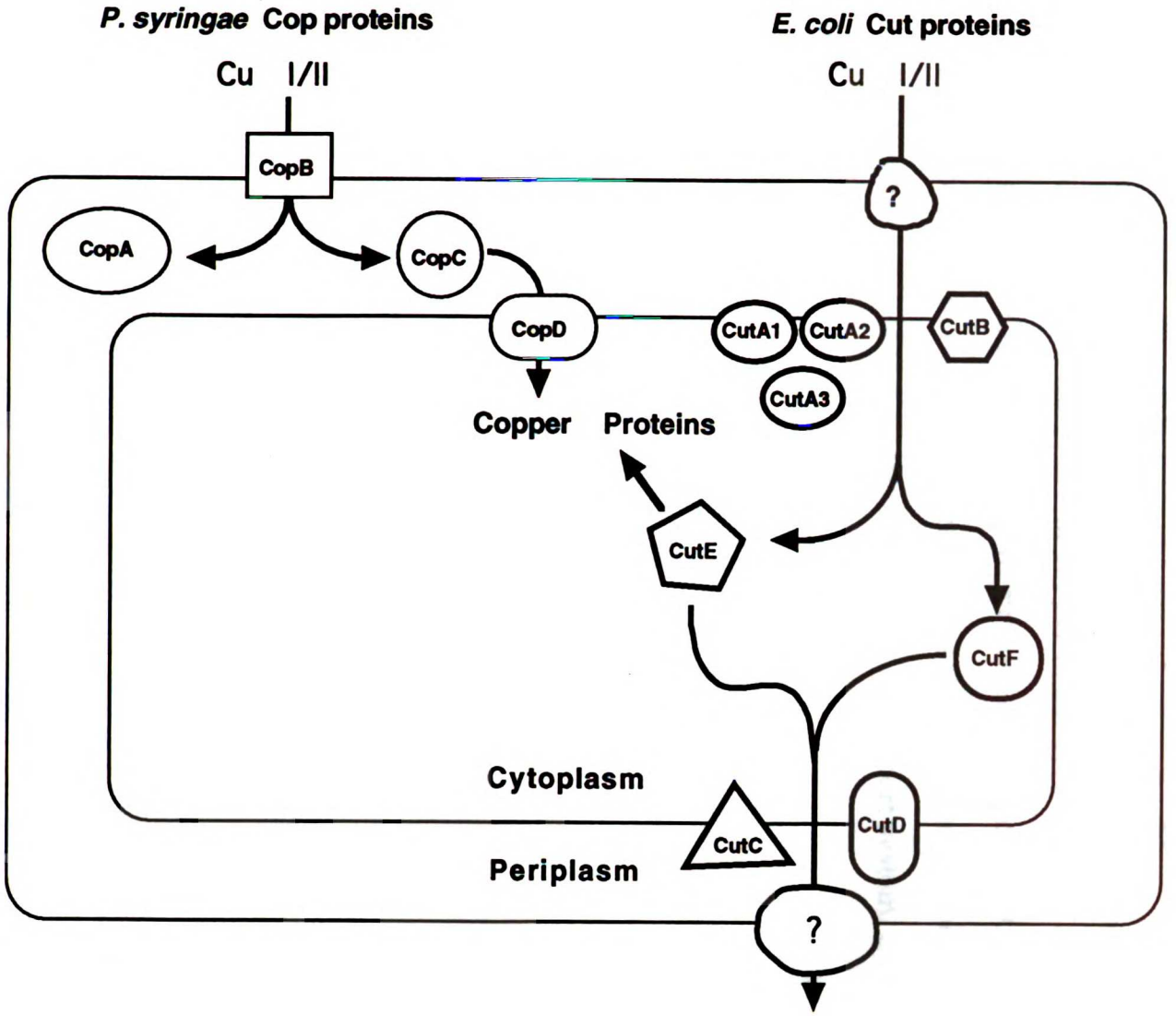

P. syringae Cop proteins
E. coli Cut proteins
Cu I/II
Cu I/II
CopB
?
CopA
CopC
CopD
CutA1
CutA2
CutB
CutA3
Copper Proteins
CutE
CutF
Cytoplasm
CutC
CutD
Periplasm
?

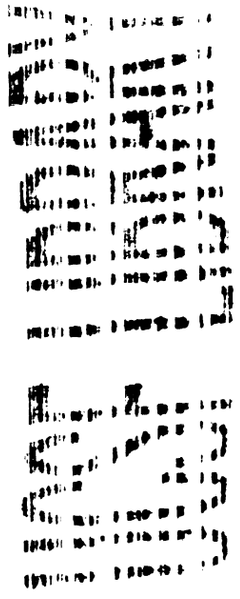
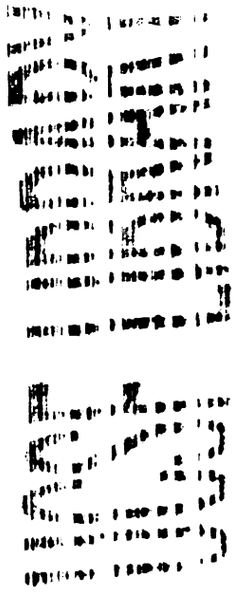


Figure 1. **EXAMPLES FROM BACTERIAL COPPER TRANSPORT**

In the model above, adapted with permission from Cooksey et al [1994] and Brown et al. [1994], CopB of *P. syringiae* transports copper into the periplasmic space, where it is bound by CopA and CopC. CopA binds copper in the periplasm, and may have both a storage and an enzymatic function. CopC and CopD cooperate in the transport of copper to the cytosol. In *E. coli*, an additional system, the Cut proteins, are depicted. Several proteins, Cut A1-3, and Cut B, cooperate in transport process across the inner membrane. Cut E and Cut F deliver copper to cuproproteins and to the export system, Cut C and Cut D.



Model of *S. cerevisiae* copper transport

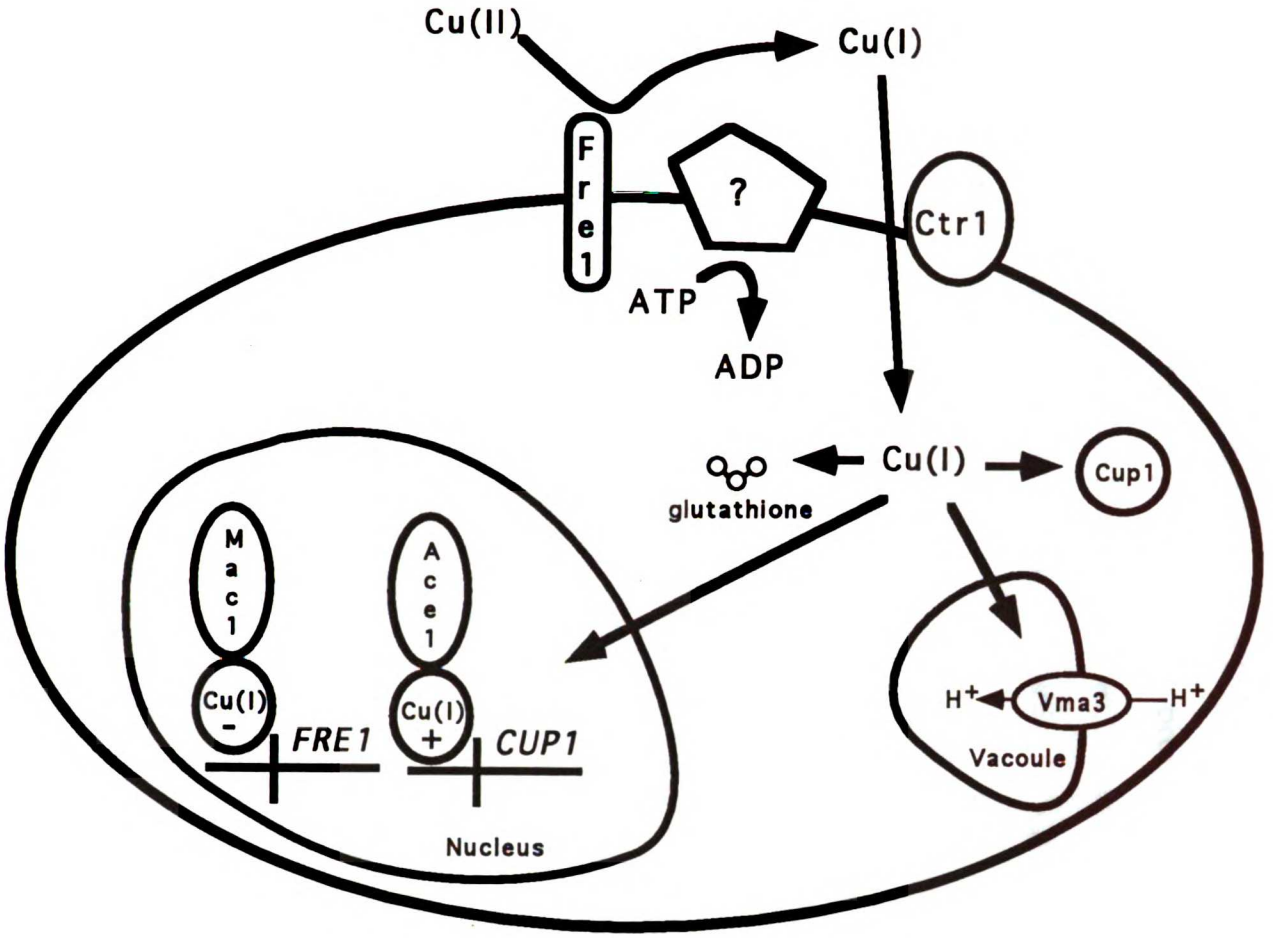
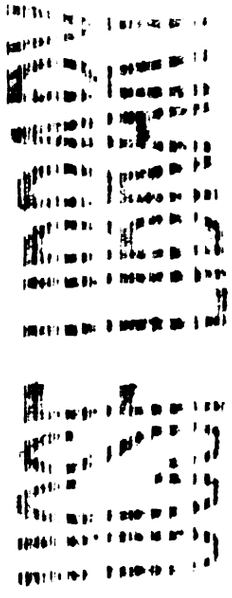


Figure 2. **MODEL OF S. CEREVISIAE COPPER TRANSPORT**

An extracellular facing reductase, Fre1, converts Cu(II) to Cu(I). Cu(I) is transported into the cell in an energy dependent process, involving at least Ctr1. Intracellular copper is bound to glutathione and Cup1 (yeast metallothionein). Ace1 and Mac1 are copper regulatory proteins, which modulate transcription of *FRE1* and *CUP1* in response to intracellular copper. Entry of copper into vacuoles is dependent on the vacuolar H^+- ATPase, of which Vma3 is a subunit.



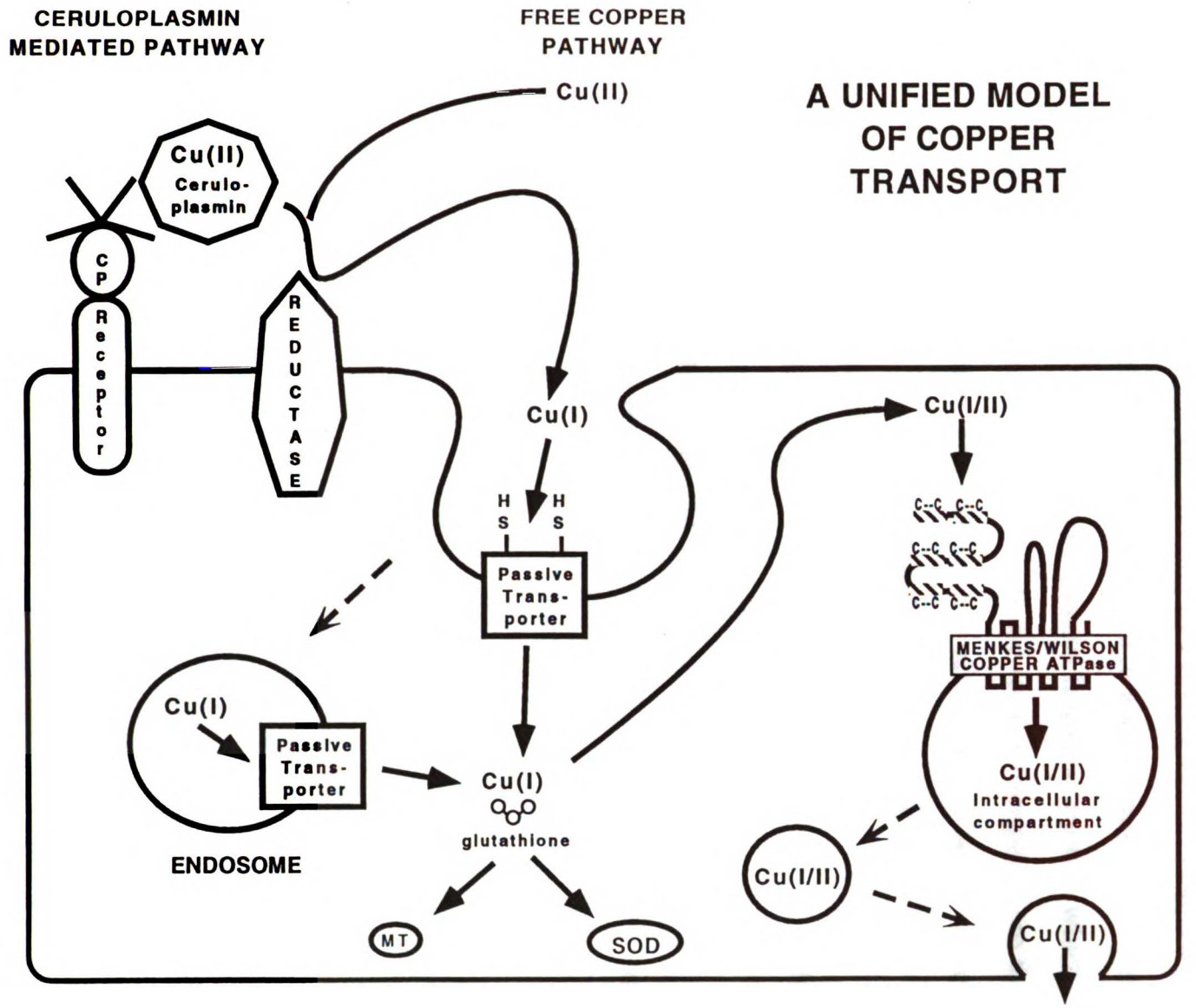
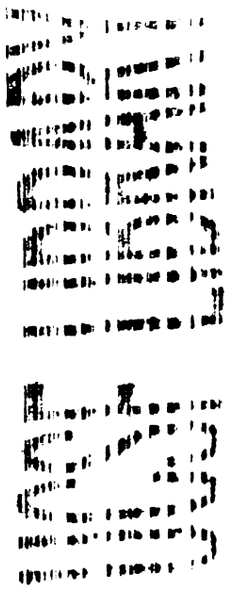

CERULOPLASMIN MEDIATED PATHWAY
FREE COPPER PATHWAY
A UNIFIED MODEL OF COPPER TRANSPORT
Cu(II)
Cu(II) Cerulo-plasmin
CP Receptor
REDUCTASE
Cu(I)
H S
H S
Passive Trans-porter
Cu(I)
Passive Trans-porter
ENDOSOME
Cu(I)
glutathione
MT
SOD
Cu(I/II)
MENKES/WILSON COPPER ATPase
Cu(I/II) Intracellular compartment
Cu(I/II)
Cu(I/II)

Figure 3. **A UNIFIED MODEL OF COPPER TRANSPORT**

The model proposes that both ceruloplasmin-mediated transport and free copper transport utilize the same cellular components in the uptake process. In ceruloplasmin-mediated transport, binding to a ceruloplasmin receptor is followed by release of copper from ceruloplasmin in a step requiring copper reduction. Cu(I) enters the cytoplasm via a passive transport protein which does not require cellular energy to function. Some copper transport into the cytosol occurs at the cell surface and some occurs after internalization of the copper-transporter complex in an endosome. Cu(I)-glutathione transfers cystosolic copper to cuproproteins, such as metallothionein (MT) or superoxide dismutase (SOD). Copper is delivered to the export system by the Menkes/Wilson copper transporting ATPase in one or more intracellular compartments/organelles. In this model, free copper or copper bound to other ligards utilizes the same reductase and follows the same path as copper from ceruloplasmin.



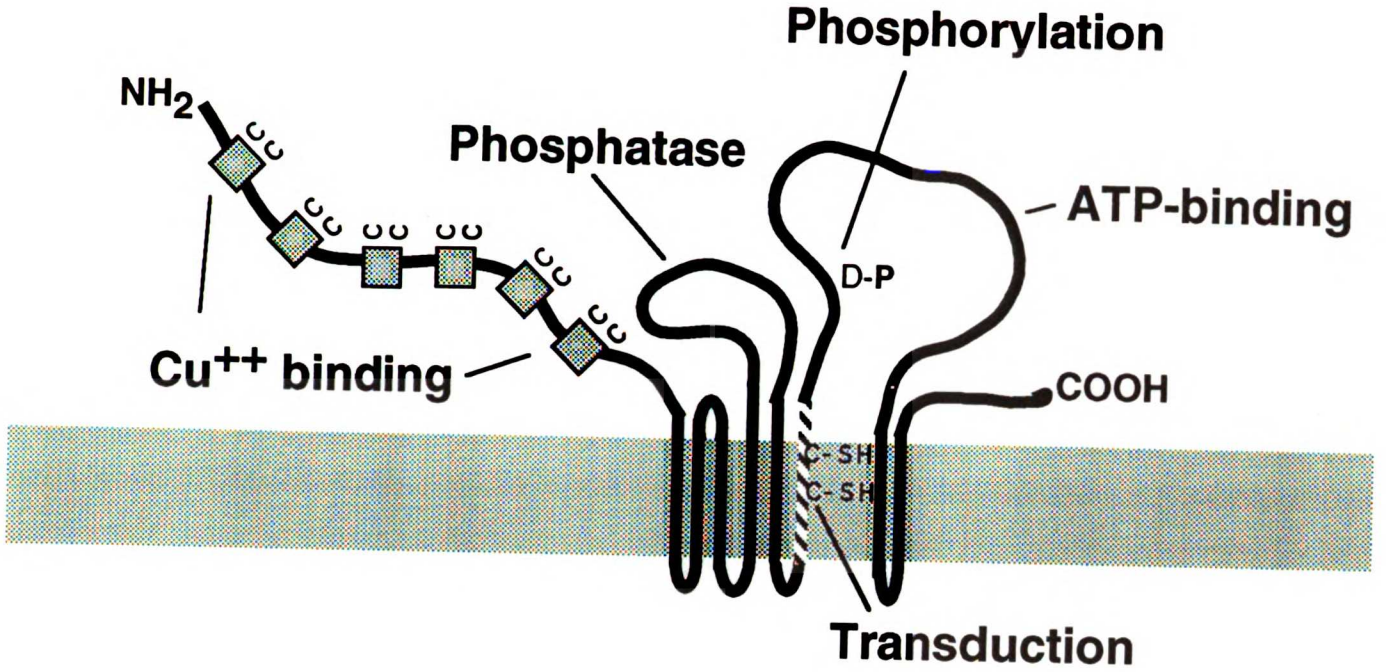
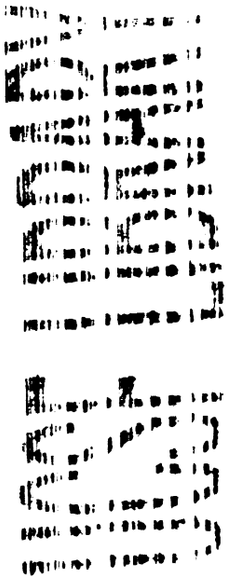

Phosphorylation
NH2
Phosphatase
ATP-binding
D-P
Cu++ binding
COOH
C-SH
C-SH
Transduction

Figure 4: Model for the proposed Menkes/Wilson copper transporting ATPase based on homology to other P-type ATPases. The boxes with the paired cysteines(C) represent the metal binding motifs.



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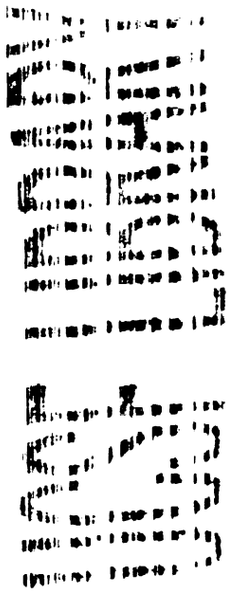

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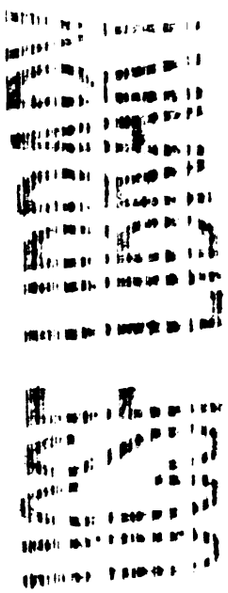

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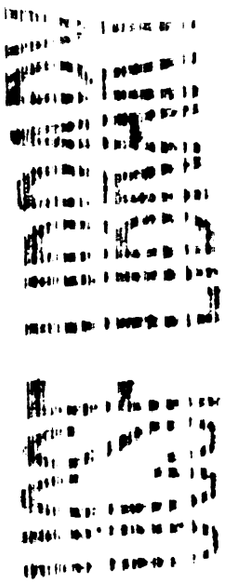

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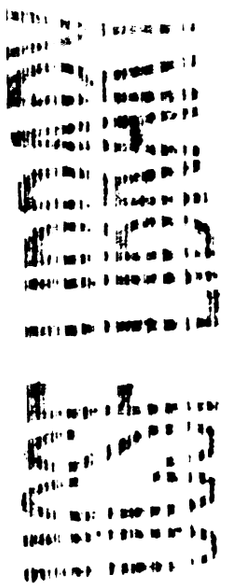

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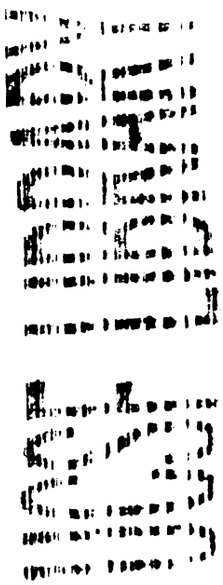

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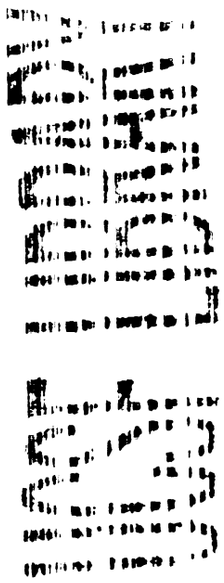

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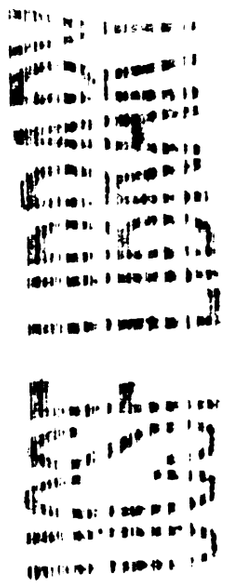

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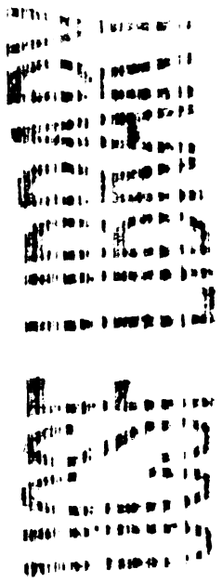

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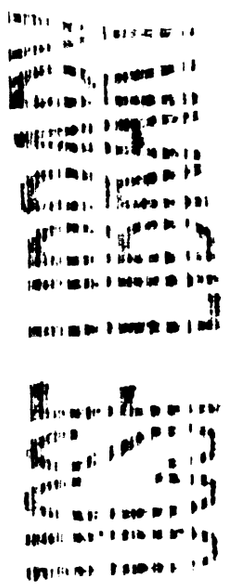

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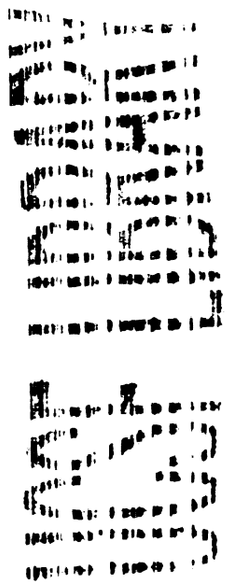

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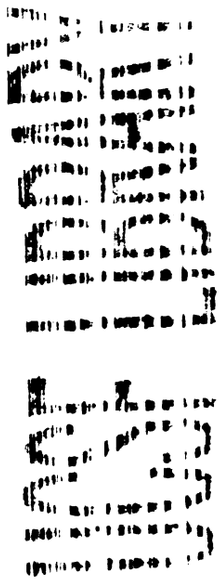

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