Journal of Medical Genetics, 1980, **17**, 262–266

Menkes X linked disease: two clonal cell populations in heterozygotes

NINA HORN, PETRA MOOY, AND V MARY McGUIRE

From the Department of Medical Genetics, John F Kennedy Institute, Glostrup, Denmark; the Department of Clinical Genetics, Erasmus University, Rotterdam, The Netherlands; and the Paediatric Research Unit, Guy's Hospital, London

SUMMARY The ^{64}Cu incorporation into fibroblast clones obtained from three obligate and three suspected Menkes disease heterozygotes was studied. For each obligate heterozygote, two clonal cell populations were observed, one with a Menkes phenotype and one with a normal phenotype, as predicted by the Lyon hypothesis. The cloning results suggested a heterozygous state in two of the suspected carriers.

The theoretical and practical limitations of the cloning method for identification of carriers of X linked diseases are discussed.

Menkes steely hair disease is a serious X linked inborn error of metabolism resulting in increased copper binding in several cell types including cultured fibroblasts and amniotic fluid cells.[1-4] The syndrome is characterised by peculiar hair, poor temperature control, vascular and bony changes, severe mental and physical retardation, and death in early childhood.[5-7]

Affected males can be identified biochemically by ^{64}Cu uptake studies in tissue culture [8 9] and prenatal diagnosis with abortion of males with the cellular defect has now been done in 40 pregnancies at risk.[10] Obviously there is a need to identify carrier females. The heterozygous females would be expected to be mosaics of wild type and mutant cells, according to the Lyon hypothesis of random X chromosome inactivation.[11] In general if a mutant X linked gene is expressed in culture at the cellular level, the resulting cellular mosaicism should be detectable. Biochemical tests usually require assay of many cells, and will show mosaicism only after cloning. In some cases the two cellular phenotypes (mutant and wild) may be detectable in vivo in heterozygotes. Phenotypic patchiness in some heterozygotes suggests that the Menkes gene, like most X linked genes, is subject to random X inactivation. A distinct mosaic phenotype has been reported in a black heterozygous girl showing variegated skin pigmentation.[12] The presence of 'kinky' patches in the scalp hair in some female carriers[7 13] can also be explained on the basis of X inactivation. The demonstration of in vitro expression of the defective Cu metabolism prompted the investigation of Cu uptake into clonal cell lines in an attempt to identify two distinct cell populations. Direct detection of the two cell populations by autoradiography is not applicable because of the short half life of the copper isotopes available. The object was firstly to obtain evidence on lyonisation of the *Menkes* locus, and secondly to test the validity of the method for carrier detection.

Received for publication 27 November 1979

Materials and methods

CELL CULTURES

Three obligate heterozygotes (BM, ZM, and LL), one sister (BB), and one maternal aunt (SM) of Menkes disease patients, and a maternal second cousin of an obligate carrier (HJ), were studied. Cell cultures from the male probands had shown incorporation values consistent with Menkes disease. Cell cultures from the heterozygotes, from two normal females, and from an affected male were established in Ham's F10 medium with 15% fetal calf serum and penicillin 100 units/ml and streptomycin 100 μg/ml. Clones were obtained from early fibroblast outgrowths by making a suspension of about 40 cells/ml and inoculating 25 μl aliquots into each well of a 96 well plate. Of a total of 124 clones from the nine subjects investigated 12 clones could

TABLE *Tissue culture studies in cloned and uncloned fibroblasts from Menkes patients, obligate and potential carriers, and controls.*

No	*Genotype*	*^{64}Cu incorporation results**		*Cloning results*				
		Culture from index case	*Uncloned culture*	*Obtained*	*Tested*	*Phenotype*		
						Menkes disease	*Normal*	*Intermediate*
1 (RP)	Menkes disease	—	79·8†	10	10	10	0	0
2	Control	—	13·0†	10	7	0	7	0
3	Control	—	22·6†	9	9	0	9	0
4 (BM)	Obligate carrier	61·7	38·6	14	14	1	10	3
5 (ZM)	Obligate carrier	70·6	53·3	14	14	8	3	3
6 (LL)	Obligate carrier	111·6	34·2	11	8	4	2	2
7 (SM)	Affected nephew	111·6	24·0	17	17	8	7	2
8 (BB)	Affected brother	71·3	15·1	12	12	0	12	0
9 (HJ)	Affected maternal second cousin	123·0	17·1	27	21	0	18	3

* 95% confidence limits for 20 controls: 12·4–31·9 ng ^{64}Cu per mg protein per 20 h. 95% confidence limits for 18 Menkes disease patients: 46·5–99·9 ng ^{64}Cu per mg protein per 20 h.
† Mean of the clones tested.

not be used, six because of poor growth and six because of fungus contamination (table).

INCORPORATION STUDIES

Studies of labelled copper (40 to 50 Ci/g Cu) uptake were performed.[8] The cells (100 000 per well) were plated in semimicro plates, and incubated for 20 hours with medium containing about 10 μmol/l $^{64}CuCl_2$. Amphotericin, shown to have no effect on Cu incorporation, was added to the test medium. The cells were then washed in 0·02 mmol/l edetic acid in physiological saline, rinsed in physiological saline, and lysed in 10% sodium hydroxide at 80°C for 10 minutes.

The radioactivity in 100 μl aliquots was counted in a liquid scintillation spectrometer (Beckman LS 100 C) with an open window. The counts were corrected for half life decay ($T\frac{1}{2}=12·8$ h) and converted to total Cu by means of standards included in the counting series. The Cu uptake was related to the protein content measured on 20 μl aliquots by a modified Lowry method.[14]

The cell lines were tested in the fourth or fifth passage after cloning. An uncloned culture established from the same skin biopsy was tested simultaneously.

In the first two tests serum from pregnant women was used as a constituent of the incorporation medium. This was, however, discontinued because the results were unreliable, and in the remaining tests fetal calf serum was used instead. In addition, the incorporation medium was changed from medium 199 to F12. The cultures were transferred to the incorporation medium at least one week before the test.

Contaminated clones (six) were not tested because fungus contamination had been shown to give rise to high values.

Results

The 20-hour incorporation values for the clonal cell lines are presented in the figure and summarised in the table. The results of the uncloned cultures from the people subjected to cloning and from the probands are also reported in the table. The precision of the method, calculated on the basis of the differences between duplicate determinations of the various clones, and expressed as the coefficient of variation, was 5·6%. This is close to that reported previously for double determinations of uncloned cultures of controls and Menkes disease patients.[8] Clones assayed from an affected male (RP) were used as positive controls. They gave values clearly in the Menkes disease range, distinctly above and well separated from the normal range. The values for the clones of the two control females were all within the range of the normal uncloned control cultures with little variation.

One obligate carrier (BM), a woman with three affected sons, had one clone with a value in the Menkes disease range and in addition three clones with intermediate values. The remaining ten clones had values in the normal range. The two other obligate carriers, each with one affected son and a positive family history, both had intermediate values for clones in addition to values in the Menkes disease and the normal ranges.

SM is the sister of LL and the cloning results gave seven normal values and ten significantly increased values (eight in the Menkes disease range) indicating that she, like her sister, is undoubtedly a carrier.

No clones from a maternal second cousin (HJ) of an obligate carrier had values in the Menkes disease range. Three clones with clearly intermediate values were, however, found indicating that she is

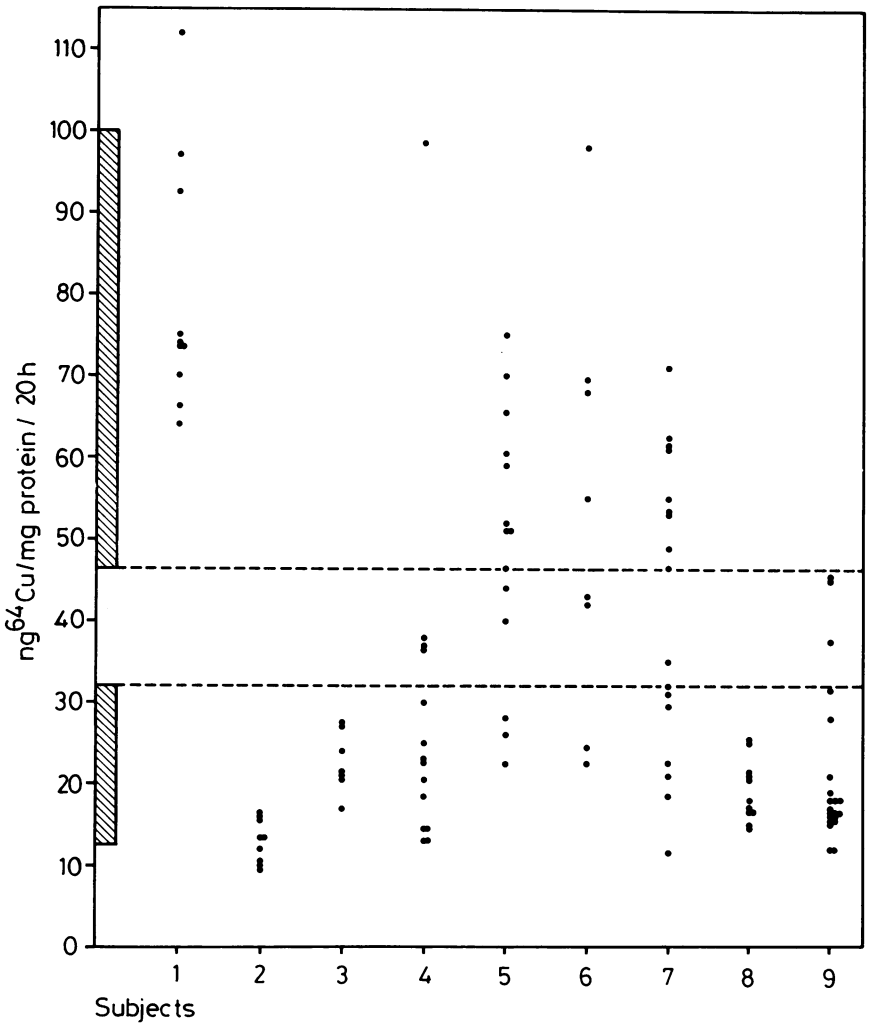

FIGURE *Clonal ^{64}Cu incorporations. Values from each subject are grouped vertically with their table designation below. The hatched bars represent the normal (lower) and Menkes disease (upper) ranges defined by the 95% confidence limits.*

also likely to be a carrier, although initially she showed normal incorporation values in an uncloned culture. A further uncloned culture from another skin biopsy of this woman showed an increased incorporation value of 47·3 ng ^{64}Cu/20 h/mg protein. The study of uncloned fibroblasts from the three obligate carriers (BM, LL, and ZM) showed incorporation values above the normal range and thus suggested heterozygosity. A culture from BB, the sister of a Menkes disease patient, had normal Cu incorporation and yielded no clones with an abnormal phenotype.

Discussion

This study confirms the expected mosaicism of Menkes disease heterozygote fibroblasts with two clonal cell populations, one type with normal Cu uptake and a mutant type with increased binding. Thus the *Menkes* locus is yet another example of an X linked gene shown to be subject to 'lyonisation'.

Some of the cloned cell cultures from each of the four proven female carriers (BM, LL, ZM, and SM) gave intermediate values (table). Since mixed cell populations of normal and mutant cells show no

metabolic co-operation in culture,[14a] it may be that these intermediate values result from mixed clones, and may also be regarded as evidence of carrier status. Evidence for this interpretation would be even stronger if it was shown that in heterozygous females the normal cell population, and hence the derived normal clones, all have low normal incorporation values. It is noteworthy in this connection that the range of incorporation values for any one particular normal uncloned cell culture is much narrower than that of all normal cell cultures tested.[14a] This is supported by the narrow ranges observed for clonal values from two females with no family history of Menkes disease. Thus, dispersion of clonal values greater than that usually observed for uncloned cultures from normal subjects may indicate the presence of mixed clones with a low proportion of mutant cells. All carriers identified by the cloning results (including HJ with the three clones showing intermediate values) showed dispersion of the clonal values, which supports this view, but further data are needed. Similarly, the diagnosis of normality in a sister of a Menkes disease patient (BB) was supported by the narrow range of values observed from twelve clearly normal clones.

The proportion of mutant cells may vary from tissue to tissue, as well as within a tissue, for example the skin, and this may be a post-lyonisation event which can influence the degree of somatic mosaicism within a heterozygote. In addition, selection of either wild type or mutant cells may occur in cell culture. Both these possibilities must be considered in tests done on either cloned or uncloned cultures.

Cloning techniques can be used for the identification of some heterozygotes for Menkes syndrome, but the carrier status can only be identified from positive clones. The technique is very time consuming, and since the plating efficiency of Menkes disease cells appears to be lower than that of normal cells,[15] the problem of obtaining sufficient clones to exclude a mosaic carrier status is obvious. For example, the twelve normal clones from the sister of a Menkes disease patient (BB, table) would only exclude a 23% mosaicism with a 0·95 confidence limit, whereas analysis of 29 clones would be needed to exclude a 10% mosaicism with a 0·95 confidence limit.[16]

This study has shown that there are significantly increased incorporation values for uncloned cells from carriers (table) and that intermediate clonal values also exist in some cases. For this reason a more extensive study of ^{64}Cu incorporation in more independent uncloned cell cultures from known and suspected carriers has been completed, showing that this simpler approach is useful.[14a]

The technical assistance of Mona Ryberg and A H Hammer is gratefully acknowledged. Professor P E Polani and Dr M Mikkelsen are thanked for valuable suggestions during preparation of the manuscript. The following colleagues referred cases for this study: Dr S Vestermark, Department of Pediatrics, Glostrup Hospital, Glostrup; Dr J J P vd Kamp, Department of Pediatrics, University Hospital, Leiden; Dr S P M vd Zee, Sittard; Dr B G A ter Haar, Genetic Counselling Unit, St Radboud Hospital, Nijmegen; Dr J Willemse and Dr J A Du-Boeff, Departments of Child Neurology and Obstetrics and Gynecology, University Hospital, Utrecht; Dr B G R Neville, Department of Paediatrics, Guy's Hospital; Dr A C Berry, Paediatric Research Unit, Guy's Hospital.

This study was supported by grants from the National Board of Social Welfare, Denmark, and the Spastics Society, England.

References

1 Danks DM, Cartwright E, Stevens EJ, Townley RRW. Menkes' kinky hair disease: further definition of the defect in copper transport. *Science* 1973;**179**:1140–2.

2 Heydorn K, Damsgaard E, Horn N, Mikkelsen M, Tygstrup I, Vestermark S, Weber J. Extra-hepatic storage of copper. A male foetus suspected of Menkes disease. *Hum Genet* 1975;**29**:171–5.

3 Horn N, Heydorn K, Damsgaard E, Tygstrup I, Vestermark S. Is Menkes syndrome a copper storage disorder? *Clin Genet* 1978;**14**:186–7.

4 Horn N, Jensen OA. Menkes syndrome: subcellular distribution of copper by an ultrastructural histochemical technique. *Ultrastructural Pathology* 1980;**1**: 237–42.

5 Menkes JH, Alter M, Steigleder GK, Weakley DR, Sung JH. A sex-linked recessive disorder with retardation of growth, peculiar hair, and focal cerebral and cerebellar degeneration. *Pediatrics* 1962;**29**:764–79.

6 Danks DM, Campbell PE, Stevens BJ, Gillespie JM, Walker-Smith J, Blomfield J, Turner B. Menkes' kinky-hair syndrome. *Lancet* 1972;**i**:1100–3.

7 Danks DM, Campbell PE, Stevens BJ, Mayne V, Cartwright E. Menkes' kinky hair syndrome. An inherited defect in copper absorption with widespread effects. *Pediatrics* 1972;**50**:188–201.

8 Horn N. Copper incorporation studies on cultured cells for prenatal diagnosis of Menkes' disease. *Lancet* 1976; **i**:1156–8.

9 Horn N, Heydorn K. Prenatal detection of Menkes' disease and copper distribution in affected foetuses. *Excerpta Medica International Congress Series* No 426 1977:56–7.

10 Horn N. Prænatal diagnostik af Menkes' sygdom. *Ugeskr Laeger* 1979;**141**/**47**:3220–2.

11 Lyon MF. Sex chromatin and gene action in the mammalian X-chromosome. *Am J Hum Genet* 1962;**14**:135–48.

12 Volpinsta EJ. Menkes kinky hair syndrome in a black infant. *Am J Dis Child* 1974;**128**:244–6.

13 Collie WR, Moore CM, Goka TJ, Howell RR. Pili torti as marker for carriers of Menkes disease. *Lancet* 1978;**i**: 607–8.

[14] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;**193**:265–75.
[14a] Horn N. Menkes X linked disease: heterozygous phenotype in uncloned fibroblast cultures. *J Med Genet* 1980;**17**: 257–61.
[15] Goka TJ, Howell RR. Copper metabolism in Menkes disease. *Monogr Hum Genet* 1978;**10**:148–55.
[16] Hook EB. Exclusion of chromosomal mosaicism: tables of 90%, 95% and 99% confidence limits and comments on use. *Am J Hum Genet* 1977;**29**:94–7.

Requests for reprints to Dr Nina Horn, Department of Medical Genetics, The John F Kennedy Institute, 7 Gl Landevej, DK 2600 Glostrup, Denmark.