HURLER'S SYNDROME: A GENETIC STUDY OF CLONES IN CELL CULTURE WITH PARTICULAR REFERENCE TO THE LYON HYPOTHESIS*‡

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Cell culture offers a means by which the genetic mucopolysaccharidoses (1) may be studied since both cellular metachromasia and intracellular mucopolysaccharide content provide reliable genetic markers in cultures of skin fibroblasts from affected individuals and heterozygous carriers (2, 3, 4). As the phenotype is evident at the cellular level in cultures from both the autosomal recessive and X-linked recessive forms, the activity of the autosomal and X-linked genes controlling the expression of the Hurler syndrome (1) can be studied.

The Lyon hypothesis (5) postulates that random inactivation of one of the two X chromosomes in normal mammalian female cells occurs early in embryogenesis. Support for this hypothesis has been obtained by Davidson et al. (6), who have demonstrated the existence of two distinct populations of cells in clones of skin fibroblasts derived from females heterozygous for two electrophoretically distinguishable mutants of the X-linked glucose 6-phosphate dehydrogenase locus. It has been suggested (7) that certain autosomal loci may be subject to the same kind of random inactivation, although this view has not yet been substantiated.

Cloning of skin fibroblasts derived from patients and heterozygous carriers for the gene for the Hurler syndrome of both the autosomal recessive and X-linked recessive types, should provide information relevant to the Lyon hypothesis.

Materials and Methods

Eight families have been studied (four X-linked recessive and four autosomal recessive). All families have been investigated clinically (8) and cytologically (2, 3, 9) with the exception of the families M.D. (X-linked recessive) and J.W. (autosomal recessive). The affected individual in each family was classified as a specific mucopolysaccharidosis according to the criteria suggested by McKusick (1).

Skin biopsies were obtained from normal subjects, patients with mucopolysaccharidoses,

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and certain of their relatives. Cell lines from the skin biopsies were established by standard culture procedures previously described in detail (2). Cell cultures to be used for cloning were grown in plastic Petri dishes in Eagle's minimum essential medium with 20% human AB serum and 5% beef embryo extract ultrafiltrate (10) in an atmosphere of 5% CO_2 in air. After several weeks during which the fibroblasts migrated from the original explants and divided, each culture was trypsinized; clones were developed from single cell platings of cultured cells according to the dilution techniques described by Puck et al. (11). The Petri dishes were not moved from the time of inoculation of single cells until the clones were ready for subculture into monolayer cultures to avoid dislodgement of cells and mixing of clones. The clones were trypsinized by the cylinder cloning technique (11), transferred to a Petri dish or to a 2 oz glass flask to establish monolayer cloned cultures, coded by letter, and grown as previously described (2).

Whenever a cloned culture was subcultured, a small cover slip was introduced into the flask to obtain a cytological preparation. 14 days after subculturing, the cover slip was removed and the cells stained with the metachromatic dye, toluidine blue O, as previously described (2). Clones were grown in 2 oz bottles and subcultured every 2 wk, at which time the cells were divided into two cultures, one for cytological study and the other kept as a maintenance culture.

In order to study a large number of clones from one mother heterozygous for the X-linked recessive form of Hurler's syndrome, 523 clones were established as monolayer cultures. One hundred clones were trypsinized once to establish monolayer cultures and then allowed to grow undisturbed for 6 wk except for a change of culture medium once a week. Concurrently, 423 clones were subcultured every 2 wk and examined cytologically for the presence of metachromasia.

After the third subculture, positive and negative metachromatically staining clones were selected and established in stock cultures for chemical analyses. Each time the clone was subcultured, all cells were transferred so that by the eighth to the tenth subculture, the clones had multiplied sufficiently to form a compact monolayer in a 32 oz culture flask. The mucopolysaccharides were isolated by the method of Wessler[1] and total polysaccharide estimated as uronic acid by the carbazole method (12).

RESULTS

Establishment of Cloned Cultures.—The cloning efficiency (Table I) averaged 18.0% (range 15.3–20.0) irrespective of the source of the skin fibroblasts. Clones were established with equal ease from normal subjects, affected individuals, and clinically normal carriers of the different mucopolysaccharidoses. Confluent clones appeared in all cloning experiments and were excluded from the results as the origin of the clones was ambiguous. In the establishment of monolayer cultures from clones, approximately 12% were lost due to poor growth (Table I). The monolayer cultures grew and remained viable through approximately 11 subcultures.

Cellular Metachromasia.—The primary cultures trypsinized to obtain cell suspensions for clonal plating showed no cellular metachromasia. All primary cultures from patients with the Hurler syndrome demonstrated cellular metachromasia after two or more subcultures as monolayer cultures. In the 62 clones derived from seven unrelated normal individuals (Table III) the fibroblasts remained ametachromatic through 11 subcultures. In the four kindreds with

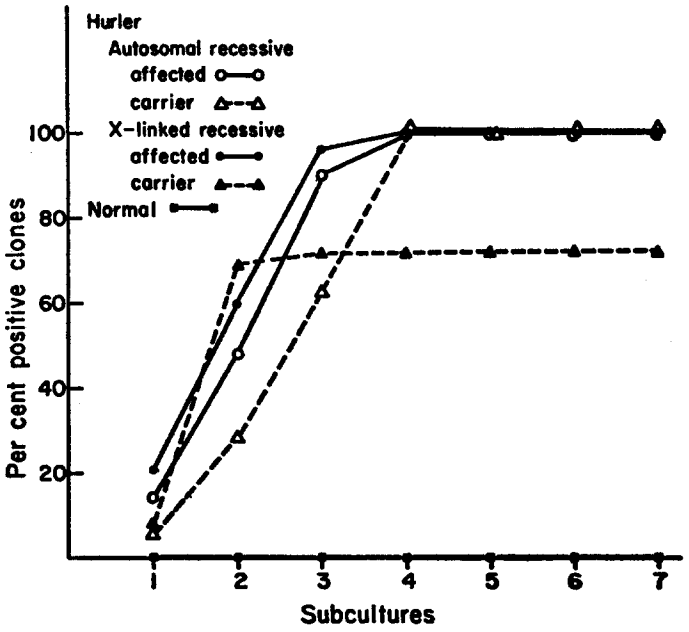
[1] Wessler, E. Unpublished data.

the autosomal recessive forms of the Hurler syndrome (Table II), clones derived from the affected individuals showed increasing metachromasia so that by the fourth subculture all the clones contained metachromatic cells (Text-fig. 1). Although the clones derived from two affected individuals (T.B. and J.E.) contained approximately the same percent of metachromatic cells as the

TABLE I

Clones of Skin Fibroblasts from a Family (M.D.) with the X-Linked Recessive Form of Hurler's Syndrome

Subject	No. cells isolated	No. clones obtained	Cloning efficiency	No. monolayer cultures obtained	Monolayer cultures efficiency
			%		%
Propositus	600	93	15.5	83	89.3
Father	600	100	16.7	84	84.0
Mother	2340	468	20.0	423	90.4
Sister 1	600	92	15.3	74	80.4
Sister 2	600	100	16.7	87	87.0
Total	4740	853	18.0	751	88.0



TEXT-FIG. 1. Time sequence (based on subcultures) of cellular metachromasia in clones of skin fibroblasts derived from normal subjects, affected individuals and heterozygotes for Hurler's syndrome.

uncloned cultures, there was more uniformity in the amount of intracellular metachromasia in the clones. Clones derived from L.R. contained only metachromatic cells although the uncloned cultures consistently had 30% ametachromatic cells. Cell density did not appear to influence significantly the time at which cellular metachromasia was detected.

The 106 clones from the six heterozygotes for the autosomal recessive forms of Hurler's syndrome all showed cellular metachromasia (Table II). The percent of positive cells remained approximately the same as in the parent cell

TABLE II

Metachromatic Granules in Skin Fibroblast Cultures from Four Families (T.B., J.W., J.E., and L.R.) with the Autosomal Recessive Forms of Hurler's Syndrome

Subjects	Cellular metachromasia (Fourth subculture)			
	Uncloned	Clones		
	Percent cells positive per culture	Total No.	Percent positive	Percent cells positive per culture
Family T.B. (Hurler)				
Propositus	89 (86–94)	12	100	95 (90–97)
Father	30 (28–35)	22	100	70 (68–75)
Mother	34 (30–38)	15	100	58 (52–60)
Family J.W. (Scheie)				
Mother	60 (55–62)	21	100	90 (88–94)
Family J.E. (Hurler)				
Propositus	82 (79–85)	24	100	90 (88–94)
Mother	38 (35–40)	10	100	60 (57–63)
Father	29 (26–32)	20	100	52 (49–55)
Paternal grandfather	34 (30–36)	18	100	80 (78–85)
Sister (noncarrier)	0	9	0	0
Family L.R. (Hurler)				
Propositus	70 (62–78)	60	100	100

lines but cellular metachromasia was more uniform. Approximately 10% of the clones had the usual diversity of metachromasia associated with cultures derived from numerous lineages and were considered to be mixed clones. In one family (J.E.), clones derived from the clinically normal noncarrier sister, remained ametachromatic throughout nine subcultures.

In the X-linked recessive kindreds (Table III), skin biopsies from the three affected individuals studied produced cultures in which 90 to 100% of the fibroblasts were packed with metachromatic material. All clones derived from the affected individuals were positive after the third subculture (Text-fig. 1) and showed striking uniformity of metachromasia. Five of the 113 clones appeared morphologically similar to clones of gargoyle cells.

The fathers in the X-linked recessive families produced uncloned cultures

containing 0–1% metachromatic cells. The 78 clones derived from these cultures were all ametachromatic and morphologically homogeneous.

Fibroblast cultures derived from 19 heterozygous females for the X-linked recessive form had an average of 40% metachromatic cells (Table IV). The

TABLE III

Metachromatic Granules in Skin Fibroblast Cultures from Seven Normal Individuals and Four Families (M.D., M.P., J.P., and D.M.) with the X-Linked Recessive Form of Hurler's Syndrome

Subjects	Cellular metachromasia (Fourth subculture)			
	Uncloned	Clones		
	Percent cells positive per culture	Total No.	Percent positive	Percent cells positive per culture
Normal individuals (average of 7)	1	62	0	0
Family M.D.				
Propositus	100	46	100	100
Father	0	43	0	0
Mother	45	382	72	100
Family M.P.				
Propositus culture 1	90	6	100	92
culture 2	94	16	100	96
culture 3	99	24	100	100
culture 4	99	9	100	98
Father	0	25	0	0
Mother culture 1	49	40	75	100
culture 2	53	8	63	100
Family J.P.				
Propositus	96	12	100	96
Father	0	10	0	0
Mother	38	13	77	100
Maternal aunt	39	7	28	100
Family D.M.				
Mother	25	22	86	100

remainder of the cells showed no detectable cellular metachromasia. The percent of cells showing metachromasia did not appear to be influenced by the age of the skin donor, time in culture after the third subculture, or other environmental conditions. The percent of cells showing metachromasia remained constant in uncloned cultures for the entire 9 months in culture. 10% of the 525 clones derived from skin fibroblast cultures from five heterozygous females appeared to be mixed for they contained the same proportion of negative and positive cells as the uncloned cultures from which they originated and were excluded from the final analyses (Tables III and VII). Of the remaining 472

clones derived from the heterozygous females, 131 (27.8%) were negative and 341 (72.2%) positive. There was no change in the metachromasia of these

TABLE IV

Metachromasia of Uncloned Skin Fibroblast Cultures Derived from Heterozygotes for the X-Linked Recessive Form of Hurler's Syndrome

Subjects*	Metachromatic cells (1000 cells counted) (Fifth subculture)
	(%)
Family M.P.‡	
Mother Biopsy 1	49
2	51
3	45
Maternal half-aunt	46
Maternal grandmother	22
Maternal great aunt	36
Maternal great grandmother	26
Family M.D.	
Mother	45
Family J.P.	
Mother	38
Maternal aunt	39
Maternal grandmother	11
Maternal cousins 1	46
2	60
3	45
Family D.M.	
Mother	25
Sister 1	42
2	31
Family F.F.‡	
Mother	66
Sister	48
Family P.O.	
Mother	33
Family D.L.	
Mother	38
Average	40

* Kinship in relation to propositus.

‡ These values previously published in reference 2.

clones from the fourth through the seventh subculture, although the positive clones showed increased metachromasia per cell the longer they were in culture.

Fibroblast cultures from one mother (Family M.D.) showed 45% positive cells in the parent culture. Of the 523 clones established (Table V), approxi-

mately 10% contained the same proportion of positive cells as the parent culture and were thus considered to be mixed. Of the 423 clones subcultured in the routine manner, 41 (10%) were mixed; of the remaining 382 clones, 274 (71.7%) were positive and 108 (28.3%) were negative for cellular metachromasia. The clones which were uniformly positive contained no ametachromatic cells (Fig. 1). The clones which were negative contained no metachromatic granules. The 100 clones allowed to grow without subculture showed the same proportion of negative to positive clones at the end of 6 wk as the clones subcultured three times in the 6 wk period.

Chemical Studies.—Approximately eight to ten subcultures were required before a single clone formed a compact monolayer in a 32 oz flask. Cell growth had essentially ceased by this time and thus it was not possible to perform

TABLE V

Clones of Skin Fibroblasts Derived from a Heterozygote (Mother in Family M.D.) of the X-linked Recessive Form of Hurler's Syndrome

Culture condition	Total No.	Clones*		
		Positive‡	Negative	Mixed
		%	%	%
3 subcultures over 6 wk period	423	64.8	25.5	9.7
Subcultured once, grown for 6 wks without subculture	100	73.0	18.0	9.0

* Metachromasia determined at 6 wks.

‡ Clones are scored positive if any cells show cytoplasmic metachromasia.

chemical analysis of a clone more than once. Analyses of the cellular mucopolysaccharides in one family (T.B.) with the autosomal recessive form of Hurler's syndrome (Table VI) indicated that clones derived from the affected individual had approximately the same amount of uronic acid as the uncloned cultures. The clones derived from the heterozygote showed a slight increase in uronic acid content which correlated with the increase in metachromasia of the clones.

In the two families (M.P. and M.D.) with the X-linked recessive form of the Hurler syndrome (Table VI), cloning had little influence on cellular uronic acid content. The clones derived from the two noncarrier fathers showed the same cellular content as the cultures from which the clones were derived. The clones from the two affected individuals contained approximately the same amount of uronic acid as their uncloned parent cultures. The clones derived from the two heterozygous mothers could be divided into two groups. The metachromatic clones contained more uronic acid per milligram of protein than the parent uncloned cultures, whereas the ametachromatic clones had a much

lower amount of uronic acid, an amount comparable to that found in cultures, either primary or cloned, of normal fibroblasts.

TABLE VI

Cellular Uronic Acid of Skin Fibroblast Cultures and Clones from Two Families (M.P. and M.D.) with the X-Linked Recessive Form and One Family (T.B.) with the Autosomal Recessive Form of Hurler's Syndrome

Subjects	Primary uncloned cell lines*,‡		Clones‡		
	Metachromasia	μg uronic acid/mg cellular protein	Code letter§	Metachromasia	μg uronic acid/mg cellular protein
	Percent cells positive per culture			Percent cells positive per culture	
Family M.P.					
Propositus	94	17.4 ± 4.0	E	100	19.1
Mother	49	15.2 ± 1.2	H	100	18.4
			A	0	3.9
			BB	0	4.8
Father	0	5.2 ± 1.4	N	0	4.6
Family M.D.					
Propositus	100	22.4 ± 3.0	DD	100	24.1
Mother	45	16.8 ± 2.3	F	100	23.4
			TT	0	4.6
			WW	0	3.1
Father	0	4.7 ± 0.4	C	0	5.1
Family T.B.					
Propositus	94	12.3 ± 2.0	G	97	12.6
Mother	34	8.4 ± 0.4	L	58	10.9

* Clones established from these cell lines.

‡ Determinations made at 8th–10th subculture.

§ Refers to letter assigned at establishment of clones.

DISCUSSION

Cloning of human diploid fibroblasts makes possible the genetic study of cell populations derived from single cells (11). Because genetic studies of skin fibroblast cultures of different mucopolysaccharidoses (2, 3, 9) had shown that cellular metachromasia and intracellular uronic acid content were efficient genetic markers, clonal populations of cells derived from patients with the

autosomal and X-linked recessive forms of Hurler's syndrome were employed to investigate the problem of gene inactivation at the cellular level.

The cloning efficiency, 18.0%, in this study (Table I) was comparable to that reported by other workers (13) for human skin fibroblasts. Cloning efficiency did not appear to depend on the intracellular content of mucopolysaccharides since the efficiency was similar in normal and affected individuals (Table I). In an attempt to minimize cell mixing (13), the clones were undisturbed for 2 wk after seeding the cell suspension. Although overgrowth during this period occurred and confluent clones were frequently seen, only 10% of the clones appeared to be of multicellular origin (Table V).

There is accumulating evidence that skin fibroblasts from affected individuals

TABLE VII

*Cellular Metachromasia of Skin Fibroblast Clones Derived from Six Heterozygotes for the Autosomal Recessive Forms and Five Heterozygotes for the X-Linked Recessive Form of Hurler's Syndrome**

Mode of inheritance	Cellular metachromasia		Total
	Negative	Positive	
	(Number of clones)		
Autosomal recessive	0	106	106
X-linked recessive	131	341	472
Total	131	447	578

* $\chi^2 = 36.85$, 1 D.F., $P < 0.001$.

and carriers must be grown in culture for many generations before an increase in intracellular mucopolysaccharide can be demonstrated by staining or chemical analysis (3). Skin fibroblasts double three to seven times in the course of establishment of the parental cell line (13); because cellular metachromasia could not be demonstrated in clones until after the first subculture into monolayer cultures, it was assumed that 10 to 13 generations were required before detectable mucopolysaccharide was stored within the cell. Thus any analysis of clonal activity based on cell markers, metachromasia and cellular uronic acid content, could not be utilized until the clones had been grown in culture for at least 15 generations (approximately three subcultures).

The Lyon hypothesis presumes that random inactivation of one of the two X chromosomes in normal female cells occurs early in embryogenesis (5). The demonstration of two distinct populations of cells in a female heterozygous for an X-linked recessive trait in which the phenotype is evident at the cellular level would be evidence favoring the Lyon hypothesis. The skin fibroblast cultures grown directly from skin biopsies from females heterozygous for the

X-linked recessive form of Hurler's syndrome contained cells with and without cytoplasmic metachromasia giving the cytological impression that two distinct cell populations were present. These uncloned cell lines derived from 19 heterozygous females for the X-linked recessive form showed an average of 40% (range 11–66%) metachromatic cells (Table IV); this proportion subsequently remained constant throughout 9 months of continuous growth as monolayer mass cultures (2, 3), indicating no selective advantage during continuous growth in culture.

Cloning a mixed culture from the female heterozygote yielded two distinct cell populations as determined by cellular metachromasia and uronic acid content (Tables III and VI). The clones of ametachromatic cells appeared to correspond both morphologically and chemically to normal fibroblasts and the metachromatic clones to the mutant type. It was apparent however that positive and negative clones were not randomly derived from the initial cell suspension. Thus, in one instance (mother in family M.D., Table III), the initial cell suspension contained 55% negative cells whereas only 28% negative cells were obtained on cloning from the suspension, indicating that selection must have taken place. Although the methods available for the establishment of cell lines and clones are beset with sampling errors and selection biases, the appearance of two distinct stable cell populations strongly supports the Lyon hypothesis (5).

Grüneberg (14) recently challenged the Lyon hypothesis (5), questioning the time of inactivation of the X chromosome. As biopsies from skin and myometrium of females heterozygous for glucose 6-phosphate dehydrogenase deficiency contained two populations of cells, those in which the normal allele was expressed and those in which the mutant allele could be demonstrated (15), he suggested X inactivation may occur late in the life cycle of differentiated cells as no major redistribution of cells in the skin or myometrium is known to occur. In the present study the biopsies from which the cell lines and clones were derived were usually less than 1 mm^3. Both metachromatic and ametachromatic cells originated from these biopsies. However, histological section of the skin did not show cellular metachromasia. It appeared that in both cell lines and clones, a period of adaptation was required before the cell marker was consistently present (Text-fig. 1). Once cellular metachromasia was established, the cells grew true to type. As previously suggested by Nance (16) and Beutler (17) the time required for adaptation may distort the quantitative aspects of gene inactivation inherent in the Lyon hypothesis (5).

Beutler (7) has raised the interesting possibility that autosomal loci may be subject to the same type of random inactivation. Thus far, cell culture studies have been unable to demonstrate autosomal inactivation at the loci controlling phosphoglucomutase (18), 6-phosphogluconic dehydrogenase, or lactic dehy-

drogenase (19). Certainly, there was no evidence in the present study to substantiate autosomal inactivation as 106 clones from six heterozygotes for the autosomal recessive forms of Hurler's syndrome were examined without finding any totally ametachromatic cloned cultures (Table II). Both on the basis of cellular metachromasia and uronic acid content (Tables II and VI), the clones resembled their parent cell lines. As was observed with the parent lines, the clones showed a spectrum of cellular metachromasia; this variability of metachromasia in cells all descendants of one cell may reflect, in part, the influence of environmental conditions and/or the sensitivity of the staining method for detecting the cell marker. Statistical analysis (Table VII) of the metachromasia of cloned populations derived from heterozygotes of the autosomal recessive forms compared to the X-linked recessive form showed that the difference was unlikely to be due to chance.

Since the evidence that one X chromosome in the female mammalian cell is inactive (5, 6), preliminary, thus far unsuccessful, attempts have been made to derepress the inactive X chromosome (20). The establishment of ametachromatic clones from females for the X-linked recessive form of the Hurler syndrome provides an additional in vitro model for studies on chromosome inactivation.

SUMMARY

Clones of skin fibroblasts from normal individuals, patients with different mucopolysaccharidoses, and certain of their relatives have been examined for cellular metachromasia and cellular uronic acid.

All the clones derived from affected individuals and heterozygous carriers in families with the autosomal forms of Hurler's syndrome showed marked metachromasia and increased cellular uronic acid. Since only one cell population was demonstrated in clones derived from heterozygous carriers, no evidence for autosomal inactivation was obtained.

Clones derived from affected individuals with the X-linked recessive form of Hurler's syndrome contained uniform populations of metachromatic staining cells which demonstrated increased cellular uronic acid. Clones derived from the noncarrier fathers showed no cellular metachromasia or increased cellular uronic acid. Clones derived from the heterozygous mothers and sisters showed two populations both qualitatively and quantitatively. On the average, 72% of these clones were metachromatic and demonstrated an increased uronic acid content; 28% of the clones showed no metachromasia and the uronic acid content was similar to that found in normal individuals.

The appearance of two distinct cell populations in clones derived from females heterozygous for the X-linked recessive form of Hurler's syndrome provides evidence in favor of the Lyon hypothesis.

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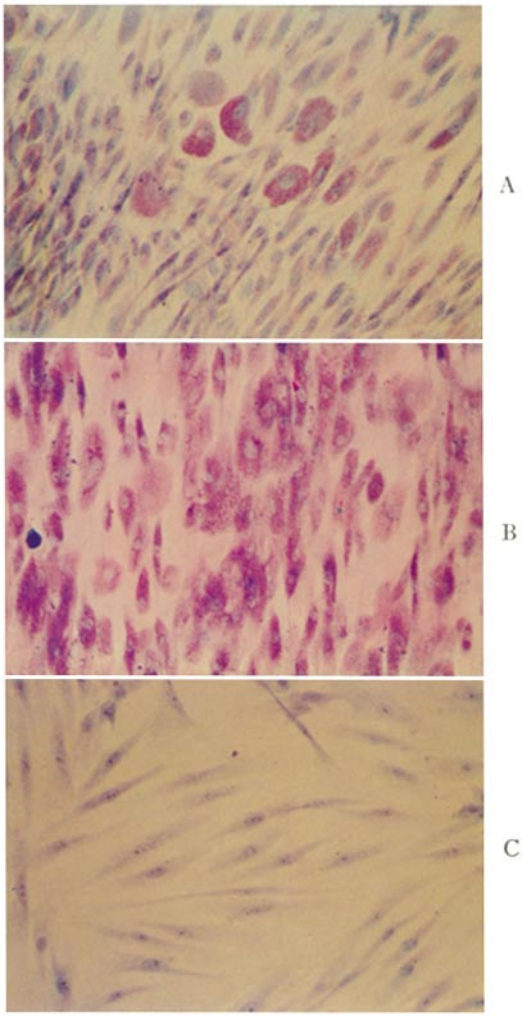
BIBLIOGRAPHY

1. McKusick, V. A., D. Kaplan, D. Wise, W. B. Hanley, S. B. Suddarth, M. E. Sevick, and A. E. Maumanee. 1965. The genetic mucopolysaccharidoses. *Medicine* **44**:445.
2. Danes, B. S., and A. G. Bearn. 1966. Hurler's syndrome, a genetic study in cell culture. *J. Exptl. Med.* **123**:1.
3. Danes, B. S., and A. G. Bearn. 1966. Hurler's syndrome, effect of retinol (vitamin A alcohol) on cellular mucopolysaccharides in cultured human skin fibroblasts. *J. Exptl. Med.* **124**:1181.
4. Matalon, R., and A. Dorfman. 1966. Hurler's syndrome: Biosynthesis of acid mucopolysaccharides in tissue culture. *Proc. Natl. Acad. Sci. U.S.* **56**:1310.
5. Lyon, M. F. 1962. Sex chromatin and gene action in the mammalian X-chromosome. *Am. J. Human Genet.* **14**:135.
6. Davidson, R. G., H. M. Nitowsky, and B. Childs. 1963. Demonstration of two populations of cells in the human female heterozygous for glucose-6-phosphate dehydrogenase variants. *Proc. Natl. Acad. Sci. U. S.* **50**:481.
7. Beutler, E. 1963. Autosomal inactivation. *Lancet* **I**:1242.
8. Berggård, I., and A. G. Bearn. 1965. The Hurler's syndrome, a biochemical and clinical study. *Am. J. Med.* **39**:221.
9. Danes, B. S., and A. G. Bearn. 1967. Cellular metachromasia, a genetic marker for studying the mucopolysaccharidoses. *Lancet* **I**:241.
10. Chu, E. H. Y., and N. H. Giles. 1959. Human chromosome complements in normal somatic cells in culture. *Am. J. Human Genet.* **11**:63.
11. Puck, T. T., P. I. Marcus, and S. J. Cieciura. 1956. Clonal growth of mammalian cells in vitro, growth characteristics of colonies from single HeLa cells with and without a "feeder" layer. *J. Exptl. Med.* **103**:273.
12. Dische, A. 1947. A new specific color reaction of hexuronic acids. *J. Biol. Chem.* **167**:189.
13. Martin, G. M., and A. Tuan. 1966. A definitive cloning technique for human fibroblast cultures. *Proc. Soc. Exptl. Biol. Med.* **123**:138.
14. Grüneberg, H. 1967. Sex-linked genes in man and the Lyon hypothesis. *Ann. Human Genet., London* **30**:239.
15. Gartler, S. M., and D. Linder. 1964. Selection in mammalian mosaic cell populations. *Cold Spring Harbor Symp. Quant. Biol.* **29**:253.
16. Nance, W. E. 1964. Genetic tests with a sex-linked marker: glucose-6-phosphate dehydrogenase. *Cold Spring Harbor Symp. Quant. Biol.* **29**:415.
17. Beutler, E. 1964. Gene inactivation: The distribution of gene products among populations of cells in heterozygous humans. *Cold Spring Harbor Symp. Quant. Biol.* **29**:261.
18. Sigman, B., and S. M. Gartler. 1966. The absence of inactivation at two autosomal loci. *Humangenetik* **2**:372.

19. Davidson, R. G., A. M. Glen-Blott, and H. Harris. 1965. Clonal study of three autosomal loci in heterozygotes. Reported at the American Society of Human Genetics, Seattle, Washington, Aug. 25–27.
20. Comings, D. E. 1966. The inactive X chromosome. *Lancet* II:1137.

EXPLANATION OF PLATE 37

FIG. 1. Skin fibroblast cultures from a female (mother of M.D.) heterozygous for the X-linked recessive form of the Hurler syndrome. Preparations stained with toluidine blue O. A. Parent cell line from which clones B and C derived. Sixth subculture. × 320. Both positive and negative metachromatic cells are present. B. Clone in sixth subculture. Uniformity of cellular metachromasia is marked. × 320. C. Clone in sixth subculture. All cells are ametachromatic. × 320.



(Danes and Bearn: Hurler's syndrome: a genetic study of clones)