SINGLE-ALLELE EXPRESSION AT AN X-LINKED HYPERURICEMIA
LOCUS IN HETEROZYGOS HUMAN CELLS*

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One type of control over genic expression involves repression of genes on all but one of the X chromosomes in somatic cells of mammals, according to the Lyon hypothesis or a counterpart of this concept termed the single-active-X hypothesis. (See refs. 3–6 for recent reviews.) The differentiation into a single "active" X and additional "inactive" X's (one in normal females) begins during cleavage. The two functional states of the X defined at that time appear to be stable during succeeding cell divisions; active X's replicate as such, and inactive X's produce only inactive replicas. The X that is active can be of maternal origin in some cells and of paternal origin in other cells of an individual. These statements imply that cells heterozygous for X-linked alleles will have the phenotype corresponding to either one allele or the other and that such single-allele expression will be manifested in clones derived from heterozygotes. Tests of this prediction can be made with clones of cultured cells that are heterozygous for X-linked genes having phenotypes which are expressed in vitro. This aspect of the single-active-X control scheme has already been demonstrated in clones of cultured human cells that were heterozygous for alleles at the X-linked loci concerned with glucose-6-phosphate dehydrogenase (G6PD) or with Hunter’s syndrome. Similar studies of additional genes will be necessary to evaluate the hypotheses correctly and to discover how the control is effected.

Primary hyperuricemia in boys is often the pathological expression of mutant alleles, Jh, of a normal X-linked gene, Jh. The immediate biochemical lesion is a deficiency in the enzyme hypoxanthine-guanine-phosphoribosyl transferase ("PRTase"; E.C. 2.4.2.8), which converts the bases hypoxanthine and guanine into their ribonucleotides. The enzyme can be indirectly demonstrated in cultured fibroblasts by autoradiography of cells grown in a medium containing tritiated hypoxanthine. Fibroblasts grown from mutant individuals incorporate little or none of the radioactive compound into their nucleic acids and have few grains of reduced silver over them in autoradiographs.

The hypothesis of X-linked inheritance of hyperuricemia is supported by the observation of the disease in males only up to the present time and by the data from five published pedigrees (and our unpublished pedigree). Additional supporting evidence which suggests linkage of the Jh locus to the X-linked locus, Xga, is found in one pedigree. Assuming X-linkage of Jh, one would expect the locus to exhibit single-allele expression. Rosenbloom et al. have described the occurrence of phenotypically normal and phenotypically mutant cells in a culture derived from the skin of a female heterozygous for the mutant Jh gene. We demonstrate here that clones of cells cultured from such heterozygotes maintain the same discreteness and are of two phenotypic classes, normal and mutant. These data provide additional support for the single-active-X hypothesis by
developed and after 6 hr of virtually slides related to cover first week until hypoxanthine (see then slips was tritiated hypoxanthine with 15% by prescribed assume in the 5 optics. No. 252 activated the cover density.

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Fig. 1.—Pedigree depicting part of a larger kindred in which the mutant jh gene was transmitted through at least four generations. Numbers refer to the strains of cells derived from the indicated donors. Individuals not having a number designation have not yet been studied with cell culture methods. Mutant males are designated by solidly filled squares, and half-filled circles denote obligate heterozygotes. Circles half-filled with dots signify females presumed to be heterozygous on the basis of the present results. The small triangle indicates a spontaneous abortion, and the diamond represents sibships that are not diagrammed in detail here.

showing that one more system, the third in which clones could be tested, yielded the expected result.

Materials and Methods.—Human subjects and cell cultures: Skin fibroblasts were cultivated from members of the pedigree shown in Figure 1 with the use of described methods. No. 252 (and his affected brother) have characteristics conforming to the syndrome described by Lesch and Nyhan. His red cells were deficient in PRTase activity. Since the mother (no. 248.1) had two affected sons but is, herself, phenotypically normal, we assume that she is heterozygous. Cultures 157 and 254 were from normal females unrelated to the kindred. The medium used, referred to as “2:1” below, and consisting of two parts Diploid Medium (Grand Island Biol. Co.) and one part F1, supplemented with 15% fetal bovine serum, was found to support more rapid growth than either of these media alone.

Autoradiography: (1) Uncloned populations: Cells were inoculated onto 22 X 22-mm cover slips in 35-mm-diameter Falcon plastic Petri dishes at about 1/8o maximal density. The medium was renewed on the second day after subculture, and radioactive hypoxanthine (see (3) below) was introduced on the third day, when the cell populations were increasing rapidly.

(2) Clones: Method I: Trypsinized cell suspensions from nearly confluent monolayer cultures were diluted to contain ten cells per milliliter, and 2-ml aliquots were distributed in 35-mm Petri dishes with 22 X 22-mm cover slips, giving an average of about ten cells per cover slip.

Method II: Aliquots (0.02 ml) of a cell suspension containing 50 cells per millilitre were placed on cover slips (18 mm in diameter) in 35-mm dishes which contained 0.1 ml of 2:1 about their perimeters to reduce evaporation. Two ml of 2:1 were added to each dish after a 3-4-hr attachment period. The medium was replaced twice weekly after the first week until labeling was done 3-4 weeks after inoculation. Clones by method I averaged 5 mm in diameter and contained about 2 X 10^4 cells, whereas clones by method II averaged about 1.5 X 10^4 cells.

(3) Labeling: Culture medium was replaced with 2 ml of 2:1 containing 5 μc/ml of tritiated hypoxanthine (Tracerlab; sp. act. 6.3 c/mmole) or 25 μc/ml (sp. act. 1.4 c/mmole) in the case of 43 method II clones of no. 248.1. Cover slips were rinsed in 0.9% NaCl after 6 hr of incubation and then fixed in absolute methanol for 10-15 min. They were then air-dried. After a 10-15-minute treatment with ice-cold 5% trichloroacetic acid, cover slips were rinsed in three changes of distilled water, dried, and mounted (cells up) on slides with a drop of Permount. Ilford K.5. emulsion was applied, exposed at -20°C and developed with standard techniques described by Prescott.

Microscopic observations: The unstained cells were first located with phase-contrast optics. If labeled, they remained visible when examined with bright-field optics but became virtually invisible if unlabeled. A standard pattern of fields sampling entire cover slips was used in scoring uncloned populations. Obvious macroscopic differences in grain
density were confirmed microscopically, whereas individual cells were scored at 128–640 magnifications. Some slides were mounted in glycerine for detailed study with a 40× oil apochromat objective.

Results.—The phase-contrast and bright-field appearances of normal cells (no. 247) after autoradiography are illustrated in Figure 2A and B. Every cell is clearly visible in uncloned populations when viewed with bright-field illumination; each cell is distinctly labeled, with the densest labeling usually over the nucleus. Densely labeled nuclei remain, but the cytoplasms become unlabeled after RNase treatment.

Mutant cells (no. 252) are almost invisible when viewed with bright-field optics after autoradiography (Fig. 2C and D), although examination with higher magnification reveals a low degree of labeling over the cells.

Cells with the mutant phenotype and normal-appearing cells were both found in uncloned populations of the obligate heterozygote no. 248.1. Figure 2E and F show a sample field in which five of the ten cells visible with phase contrast became almost invisible with bright field. Similar cells were found in uncloned populations of three other females (nos. 257, 258, and 259) from the kindred, each of whom is potentially heterozygous.

The two distinct cell types were used as scoring standards and in discerning a "plus-minus" (±) category of cells. The ± cells had an apparent excess of silver grains when compared with standard mutant cells (the grains were numerous

![Fig. 2. Autoradiographic appearance of cultured fibroblasts after growth in the presence of tritiated hypoxanthine. (A), (C), and (E) Phase-contrast views of cells whose bright-field appearances are shown in (B), (D), and (F), respectively. (A, B) Cell strain 247 (normal); (C, D) 252 (mutant); (E, F) 248.1 (heterozygous). Pointers in (E) indicate heterozygous cells with the mutant phenotype, which are visible with phase contrast but not with bright field (111.55×.)](image-url)
enough to make the cells visible in bright field but did not define the cell outlines as in standard normal cells). The frequencies of the three categories of cells in various strains are given in Table 1. Cells of the ± type occurred only in the four strains derived from proved or potential heterozygotes. Cultures from controls or mutant males contained only one cell type.

The clonal continuity of phenotypically mutant and of phenotypically normal cells was established by growing clones from one proved and two potentially heterozygous females and from one female control. The results are presented in Table 2.

Two types of clones were derived from the proved (no. 248.1) and presumptively (nos. 258 and 259) heterozygous strains. One type of clone was scored minus (−) because all individual cells were so lightly labeled as to place them in the − or ± class; an abundance of cells could be seen in these clones (Fig. 3A and C), but the same cells were almost invisible when viewed with bright-field illumination (Fig. 3B and D). The second type of clone was distinctly plus (+). Figure 3E and F illustrate the dense labeling of these + cells. Macroscopically these + clones are dark gray (Fig. 3G), whereas negative clones are transparent when viewed in this manner.

### Table 2. Autoradiographic phenotypes of clones of fibroblasts cultured from control and experimental females.

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Genotype</th>
<th>(+) Clone</th>
<th>(−) Clone*</th>
<th>Total</th>
<th>(−) Clone (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Method I:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>248.1</td>
<td>Jh/jh</td>
<td>78</td>
<td>61</td>
<td>139</td>
<td>44</td>
</tr>
<tr>
<td>258</td>
<td>Jh/jh</td>
<td>124</td>
<td>99</td>
<td>223</td>
<td>44</td>
</tr>
<tr>
<td>259</td>
<td>Jh/jh</td>
<td>106</td>
<td>72</td>
<td>178</td>
<td>40</td>
</tr>
<tr>
<td>157</td>
<td>Jh/jh</td>
<td>221</td>
<td>0</td>
<td>221</td>
<td>0</td>
</tr>
<tr>
<td>Method II:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>248.1</td>
<td>Jh/jh</td>
<td>13</td>
<td>30</td>
<td>43†</td>
<td>69†</td>
</tr>
</tbody>
</table>

* (−) Clones contain mainly − cells and lack + cells, but they do have some ± cells.
† These numbers apply only to cover slips having purely + or − growths. In addition, nine cover slips had growths with + and − sectors (see text).
Fig. 3.—Autoradiographic appearance of heterozygous clones after growth in the presence of tritiated hypoxanthine. (A), (C), and (E) Phase-contrast views of clones whose bright-field appearances are shown in (B), (D), and (F), respectively. (A, B) — Clone of strain 248.1; (C, D) — Clone of strain 258; (E, F) + Clone of strain 258. These clones were produced by method I (see text) (A–F: 14.55X). (G) Macroscopic view of a + clone of strain 248.1 produced with method II (see text) (1.94X). (H) Bright-field appearance of cells in a − clone of no. 248.1. Note the concentration of label over the nuclei of two ± cells (496.64). (I) Bright-field appearance of cells from a + clone of no. 248.1 (496.64X).

Nine cover slips in the cloning experiment with method II bore cell populations consisting of large + and − sectors that obviously represented clones which had originated from two separate cells and had become confluent. The number of such cover slips agrees with expectation based on the proportions of purely + and purely − cover slips in the experiment and the probability (Poisson) of drops containing two cells initially. This implies that some growths scored as being purely + or purely − actually originated from two cells of the same phenotypic class. The proportions of + and − clones given in Table 2 are to be regarded, therefore, as merely reasonably close estimates.

Individual ± and + cells from separate clones of a heterozygote are compared
in Figure 3H and I. No cell having a degree of labeling approaching that found in standard + cells has yet been found in − clones.

All 221 clones from a control female strain (no. 157) were unmistakably +.

Discussion.—The results demonstrate clonal, single-allele expression at the Jh locus in cultured, heterozygous cells. Similar demonstrations have been made with cells heterozygous for alleles at the loci concerned with G6PD.8 and Hunter’s syndrome,9 the only other genes that have been tested. These three loci are not functionally related in any obvious way. Data concerning their linkage relation within the X chromosome are still scanty.17, 21 Conceivably, single-allele expression is an attribute of a particular segment of the X containing the three loci, but it cannot be an attribute of only one of them.22

The frequency with which activation of the inactive alleles occurs in cultured cells can now be estimated with greater accuracy than previously. Earlier studies of G6PD in cultured, heterozygous cells utilized alleles causing electrophoretic variation7, 8 or a deficiency4 in enzyme activity. Activation could have been manifested by expression of both alleles in individual cells or by reversal of their roles as expressed and unexpressed alleles. A 5 per cent admixture of these cells probably could not have been detected in single clones because of the high background activity possessed by the predominant type of cell. Estimates of activation based on enzyme activities will be more sensitive where the activity of single cells can be evaluated. A single-cell method capable of detecting + cells in G6PD − clones has been described.23 In principle, single mutant cells in initially normal clones of cells heterozygous for the gene causing Hunter’s syndrome8 can also be detected, but heterozygous cells in which both alleles are expressed may have a phenotype that is not distinguishable from normal. The Jh alleles used here seem to be ideal for studying activation at the single-cell level since no clearly + cells have so far been detected in cell populations from mutant males. Should any + cells be found in such cultures, they will provide an estimate of the fraction of + cells in − heterozygous clones that can be attributed to mutational changes of the jh allele on the active X. Any significant excess of + cells above this mutational background in clonal cultures from heterozygous females could then be attributed to activation of the Jh allele. No clearly + cells have been found in 30 − clones of a proved heterozygote. The estimated average number of cells in these clones is about 1.5 × 106, which suggests an upper limit of less than 10−6 per cell division for the activation rate. This stability in the functional differentiation of the respective active and inactive X’s will be a favorable factor in experimental attempts at activation.

Our results suggest that, by observing uncloned cultures, it will be possible to identify heterozygous females who may produce affected sons and (through their daughters) affected grandsons. Caution must be exercised, however, since the incidence of − and ± cells in heterozygous cell populations is much less than the incidence of − clones. This discrepancy cannot be accounted for solely by selection during cloning. The cloning efficiency of strain no. 248.1 with method II is estimated at 0.52. The summed proportion of − and ± cells in the population from which the clones were derived was 0.14. No more than 17 cover slips should have had − clones if the cloning efficiency was one. Instead, at least 39
cells formed — clones on 39 different cover slips. Similar arguments apply to clones derived by method I, where the cloning efficiencies ranged between 0.35 and 0.60.

The phenotype studied here may not be completely cell-limited under our conditions. Phenotypically + cells in heterozygous populations may convert hypoxanthine into forms that can be incorporated by — cells. Such cross-feeding would not be possible with single clones developing in isolation and would be less efficient even where several clones develop in the same dish.

Cross-feeding also may have contributed to the difference between the low incidence of — cells that we found in heterozygous cell populations and the 50–60 per cent incidence reported by Rosenbloom et al.14 Although a large variance in the proportions of the two phenotypic classes of cells in different heterozygotes is expected because of the apparently random nature of the initial events in X-chromosome differentiation (e.g., see refs. 7 and 24), this does not appear to be the correct explanation for the difference noted here. Our work with clones indicates that the heterozygous cultures studied by us and by Rosenbloom et al. probably have similar frequencies of intrinsically — cells. The difference in the observed frequencies of — cells probably results from differences in the techniques used in the two laboratories. Our cell populations were deliberately grown so as to be in rapid, log-phase growth when labeled. This was reflected in the high proportion of nuclei that retained label after treatment with RNase. Rosenbloom et al. labeled their cells about 12 hours after subculture, before rapid cell increase had begun. RNase removed almost all label from their cells. Cross-feeding may have been minimized under their conditions. It should be possible to standardize the labeling conditions with the use of cells from obligate heterozygotes so that uncloned populations could be used for detecting heterozygosis. There will remain the chance that a very low incidence of — cells will not constitute reliable enough evidence of heterozygosity (e.g. no. 257 in Table 1); in such cases, cloning studies may be needed.

A low but definite degree of labeling occurs in cells from mutant males and — clones derived from heterozygous females. This might be the result of a utilizable radioactive contaminant in the hypoxanthine. Alternatively, such cells may have small amounts of PRTase. Comparisons of the enzyme activities of — clones derived from mutant males and from heterozygous females will provide an opportunity to detect limited expression of the allele on the inactive X. Perhaps such partial activation is already evident in the ± cells that we have observed in — clones under conditions where cross-feeding by + cells could not have occurred. These cells, two of which are illustrated in Figure 3H, may reflect either transient expression that can occur in every cell of a heterozygous — clone, perhaps at a particular part of the cell cycle, or more regular expression in a minority component of the cell population in which the functional differentiation between the X's has been partially reversed. If the reversal is stable, it may be possible to demonstrate a clonal distribution of ± cells in heterozygous — clones.

Summary.—Cultured clones of human cells that were heterozygous at an X-linked hyperuricemia locus had either the normal phenotype or the phenotype corresponding to the recessive mutant allele. These results support the single-
active-X hypothesis by showing that a third gene on the human X chromosome (in addition to those conditioning Hunter's syndrome and G6PD) manifests clonal, single-allele expression.

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† Predoctoral trainee supported by training grant GM0398 from the National Institutes of Health.
‡ Postdoctoral fellow of the USPHS.
10 We do not know of a generally accepted symbol for this locus. The symbol *jh*, signifying juvenile hyperuricemia, is used here for the recessive mutant alleles of a dominant, normal gene, denoted by *Jh*.