*Am J Hum Genet* 33:495–512, 1981

# Assignment of Genes to the Human X Chromosome by the Two-Dimensional Electrophoretic Analysis of Total Cell Proteins from Rodent-Human Somatic Cell Hybrids

DAVID R. COX,[1] UTA FRANCKE,[2] AND CHARLES J. EPSTEIN[1,3]

SUMMARY

The technique of two-dimensional (2-D) gel electrophoresis was used to identify five human X-linked gene products in crude cell extracts of mouse-human and Chinese hamster-human somatic cell hybrids. The human origin of these five polypeptides was demonstrated by their comigration with human fibroblast proteins and their failure to comigrate with polypeptides in extracts from the mouse or hamster parental cells. All five polypeptides were present in extracts of rodent-human hybrids that contained a human X chromosome, but were not found in extracts of cells that lacked a human X chromosome. Chromosome analysis of the hybrid clones revealed that the human X chromosome is both necessary and sufficient for the expression of the five polypeptides, designated pX-24, pX-27, pX-37, pX-40, and pX-56. pX-56 can be identified as the human X-linked enzyme glucose-6-phosphate dehydrogenase (G6PD) (E.C.1.1.1.49), while polypeptides pX-24, pX-27, pX-37, and pX-40 have molecular properties unlike those of known human X-linked gene products. pX-24 appears to be a membrane-bound protein that maps to the distal portion of the long arm of the human X chromosome, while pX-27, pX-37, and pX-40 are soluble proteins that map to the proximal long arm or to the short arm of the human X chromosome. 2-D gel electrophoretic analysis of extracts from somatic cell hybrids provides a general method for identifying polypeptides in crude cell extracts coded for by any specific chromosome and can be used to study primary gene products not previously amenable to genetic analysis.

Received July 22, 1980; revised December 2, 1980.

This study was supported by training grant GM-07085 and research grants GM-24309 and GM-26105 from the National Institutes of Health. C. J. Epstein is an investigator of the Howard Hughes Medical Institute.

[1] Department of Pediatrics, University of California, San Francisco, CA 94143.

[2] Department of Human Genetics, Yale University, New Haven, CT 06510.

[3] Department of Biochemistry and Biophysics, University of California, San Francisco.

## INTRODUCTION

The establishment of linkage relationships between human genes expressed in cultured somatic cells has been greatly facilitated through the use of rodent-human somatic cell hybrids segregating human chromosomes. Concordant segregation of human chromosomes with species-specific forms of human gene products in such hybrids has allowed the assignment of over 150 human gene loci to specific chromosomes [1]. Human gene products have most commonly been detected in hybrid cells by electrophoresis followed by histochemical enzyme staining [2, 3]. While this method of detection has been very useful, it is relatively insensitive [4] and can be used to study only a limited number of human gene loci.

Two-dimensional (2-D) polyacrylamide gel electrophoresis now offers a method for the study of many additional human primary gene products that previously have not been amenable to genetic analysis. This technique separates complex mixtures of protein in one dimension by isoelectric focusing and in a second dimension by electrophoresis in sodium dodecyl sulfate containing polyacrylamide gels [5]. 2-D gel electrophoresis is exquisitely sensitive and can be used to identify proteins independent of their function [6–8]. Recently, a 2-D gel electrophoretic analysis of polypeptides synthesized by a Chinese hamster × human somatic cell hybrid clone containing a single human chromosome was used to identify polypeptides coded for by human chromosome 12 [9].

We have combined the techniques of 2-D gel electrophoresis and somatic cell hybridization to develop a general method for identifying polypeptides in crude cell extracts that are coded for by any specific human chromosome. Here, we have used 2-D gel electrophoretic analysis of radioactive extracts from mouse-human and Chinese hamster-human hybrids segregating human chromosomes to identify five proteins that are coded for by the human X chromosome.

## MATERIALS AND METHODS

### *Parental Cells*

Human parental cells were either fresh leukocytes, obtained from a carrier of the balanced reciprocal translocation t(X;14)(p22;q21) or diploid human skin fibroblasts. Mouse and Chinese hamster recipient cell lines were A9 [10] and 380-6 [11], respectively. Both rodent lines are deficient in the X-linked enzyme hypoxanthine phosphoribosyltransferase (HPRT) (E.C.2.4.2.8). Human fibroblasts were maintained in monolayer culture in DME medium consisting of Dulbecco's modified Eagle's medium (Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 $\mu$g/ml). The rodent cells were maintained in monolayer culture in DME medium containing $2 \times 10^{-5}$ M 8-azaguanine.

### *Isolation and Characterization of Somatic Cell Hybrids*

Human fibroblasts were mixed in a 1:2 ratio with mouse A9 cells and fused in monolayer with 50% polyethylene glycol, mol. wt. 6,000 (J. T. Baker, Phillipsburg, N.J.) [12]. Mouse-human hybrid clones were isolated in DME medium containing 100 $\mu$M hypoxanthine, 0.4 $\mu$M aminopterin, and 16 $\mu$M thymidine (HAT), plus 1 $\mu$M oubain. Human leukocytes or human fibroblasts were fused with 380-6 hamster cells and selected in HAT to form hamster-human hybrids. The derivation of these primary hamster-human clones has been described

[11, 13, 14]. Two primary hamster-human hybrid clones (XIII-7A and XIII-4D) that had retained the human der(X)t(X;14) chromosome, one primary hamster-human hybrid (CH3-1) that retained a normal human X, and two primary mouse-human hybrids (A9498-1 and A9X) were chosen for further study. The der(X)t(X;14) chromosome present in clones XIII-7A and XIII-4D has been described in detail [11]. It consists of the entire human X chromosome, except for the telomere region of Xp, with region 14q21→14qter of human chromosome 14 translocated to the short arm of the X. Primary clones were back selected in 8-azaguanine and subcloned as described [13]. The 8-azaguanine-resistant derivatives were called "aza" clones. Here we have used nine rodent-human hybrid clones derived from five independent fusion events. Hybrid cells went through no more than two passages between karyotyping and harvesting for 2-D gel electrophoresis. Chromosomes were identified by standard trypsin/Giemsa banding methods [11] and by alkaline Giemsa staining [15]. Fifteen to 25 metaphases from each clone were analyzed in detail. Electrophoretic analysis of human and rodent isozymes was performed using Cellogel electrophoresis, as described [3].

*Radiolabeling and Lysate Preparation*

Cells were seeded in microtest tissue culture wells (Falcon Plastics, Cockeysville, Md., #3040, 6.5-mm diameter wells) at a concentration of $1 \times 10^5$ cells/well in DME or HAT medium, and incubated in 37° C for 12 hrs. The medium was then replaced with 100 μl/well of a similar medium containing 200 μCi/ml of a [$^{14}$C]amino acid mixture (Amersham-Searle, Arlington Heights, Ill., CFB.104), and the cells were labeled at 37° C for 24 hrs. Radioactive lysates were prepared by removing the radioactive medium from each well, washing each well three times in phosphate buffered saline, and adding 20–30 μl/well of O'Farrell's lysis buffer A [6]. Each lysate was centrifuged at 10,000 *g* for 5 min in a Beckman microfuge B to remove unlysed nuclei, and was then stored at −80° C.

*2-D Gel Electrophoresis*

2-D gel electrophoresis was performed according to the general procedure of O'Farrell et al. [16]. Nonequilibrium pH gradient electrophoresis (NEPHGE) gels were used for the first dimension, and were prepared using pH 3.5–10 ampholines (LKB, Rockville, Md.). A sample consisting of 5–20 μl of radioactive cell lysate containing $1–2 \times 10^5$ cpm of trichloroacetic acid precipitable [$^{14}$C]-labeled protein, and less than 10 μg of total protein, was loaded onto each first-dimension gel. In any single experiment, identical amounts of radioactivity were applied to each gel. The NEPHGE first-dimension gels were electrophoresed from 4–8 hrs at 400 V. In some experiments, isoelectric focusing (IEF) gels, prepared according to O'Farrell [6], were used for the first dimension. IEF was carried out for 18 hrs at 400 V, followed by 2 hrs at 800 V. The second dimension of electrophoresis utilized a 4.5% polyacrylamide stacking gel and a 10% polyacrylamide running gel. When electrophoresis was completed, the gels were fixed and stained with Coomassie Blue R (0.01% in 50% trichloroacetic acid) for 30 min followed by destaining in several changes of 7% acetic acid. Stained gels were dried under vacuum and exposed for 10–20 days to Kodak NS-2T no screen X-ray film, which was then processed according to standard procedures. Autoradiograms were analyzed by visual comparison. For determining the pH gradient formed in the first dimension, a parallel first-dimension gel with a normal sample of focused proteins was extruded immediately after electrophoresis and rapidly sliced into 5-mm sections. Each section was then equilibrated by gentle shaking in capped tubes containing 2 ml of extensively degassed water per tube. Measurement of pH was performed 10–30 min later. The second-dimension gels were calibrated with the following molecular weight standards: the β subunit from *E. coli* RNA polymerase (155,000 daltons); bovine serum albumin (68,000 daltons); the α subunit from *E. coli* RNA polymerase (39,000 daltons); and soybean trypsin inhibitor (21,500 daltons). Purified human erythrocyte G6PD-B was obtained from Dr. A. Yoshida (City of Hope National Medical Center), and purified human HPRT was provided by Dr. R. Palmour (University of California, Berkeley).

*Subcellular Fractionation*

Cells were labeled with [$^{14}$C]amino acids and washed as described above. To each well of cells, 100 μl of a solution containing 0.05% trypsin was added for 10 min at 37° C, and the cells from four wells were then pooled and centrifuged at 10,000 *g* for 4 min. The cell pellet was resuspended in 100 μl phosphate buffered saline, equally divided into two tubes, and recentrifuged at 10,000 *g* for 4 min. The cell pellet in each tube was then resuspended in 35 μl of water. To one of the tubes, 35 μl of O'Farrell's lysis buffer A [6] was added, and this sample was used as the unfractionated total cell extract. The other tube was rapidly frozen and thawed four times, and centrifuged at 10,000 *g* for 5 min. The supernatant was removed and added to 35 μl of O'Farrell's lysis buffer A, and this sample was used as the crude cytoplasmic fraction. The remaining pellet was suspended in 35 μl of lysis buffer A, and used as the crude membrane fraction. Equal amounts of trichloroacetic acid-precipitable radioactivity from the crude membrane fraction, the crude cytoplasmic fraction, and the total cell extract were then analyzed by 2-D electrophoresis.

## RESULTS

### *Formation and Characterization of Somatic Cell Hybrids*

Mouse-human and hamster-human hybrid clones retaining a human X chromosome were produced by fusing human cells with either mouse or hamster cells deficient in HPRT and selecting in hypoxanthine/aminopterin/thymidine medium. Each rodent-human primary clone was then recloned in medium containing either 8-azaguanine or hypoxanthine/aminopterin/thymidine to generate a pair of secondary clones, one with and the other without the human X. Four such pairs of secondary clones, derived from four independent rodent-human primary clones, were then karyotyped to determine if any human autosomal chromosome had segregated concordantly with the human X chromosome in these clones. The karyotypes of a pair of mouse-human hybrid clones, and a pair of hamster-human hybrid clones are shown in figures 1 and 2, respectively. Table 1 shows, when the karyotypes of all four pairs of rodent-human hybrid clones are compared, there is no human autosomal chromosome that consistently segregates with the human X chromosome, or which is consistently present in association with the human X chromosome.

### *2-D Gel Analysis of Human and Rodent Polypeptides*

To determine what fraction of human polypeptides can be distinguished from hamster or mouse proteins by the 2-D gel technique, radioactive extracts of the hamster or mouse parental cells were mixed with an equal amount of radioactivity of radioactive human fibroblast extract, and 2-D gels of these mixtures were visually compared with gels containing hamster, mouse, or human fibroblast proteins alone. This analysis revealed that of the 400 human polypeptides routinely identified, approximately 60 were distinguishable from mouse proteins but not from hamster proteins, 60 were distinguishable from hamster proteins but not mouse proteins, and 60 could be distinguished from both mouse and hamster proteins. These results suggest that with the use of both hamster-human and mouse-human hybrid clones, it should be possible to assign 180 of the 400 human polypeptides to specific human chromosomes.

*2-D Gel Analysis of Somatic Cell Hybrid Clones*

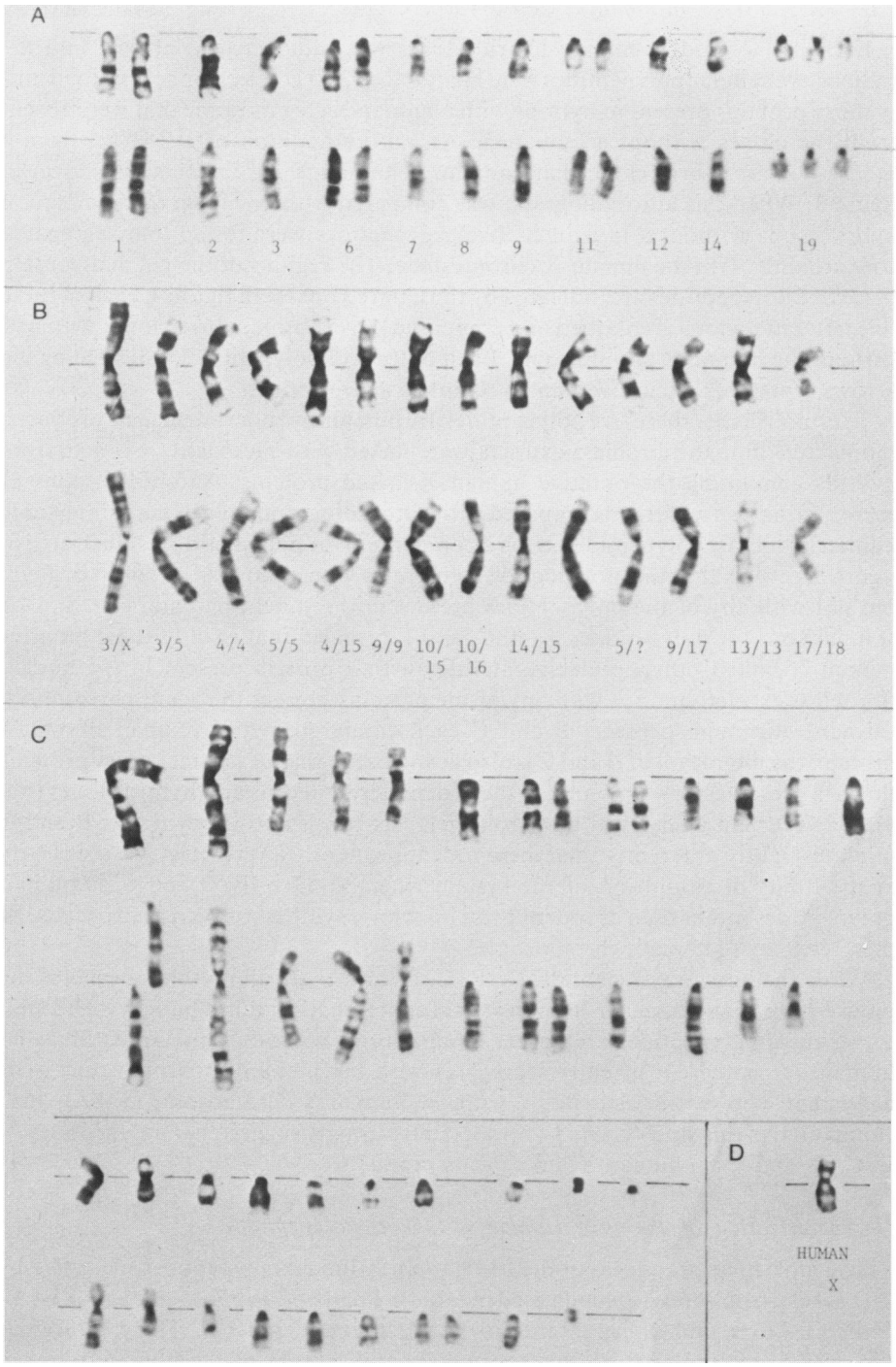
Each pair of rodent-human hybrid clones was radioactively labeled, and the radioactive cellular polypeptides were analyzed by 2-D gel electrophoresis to identify those proteins present in hybrids with a human X chromosome that were absent from the clones without a human X. An autoradiograph of a 2-D gel from a mouse-human hybrid clone that contains a human X chromosome is shown in figure 3. When this autoradiograph was compared with one from the companion hybrid clone without a human X, five polypeptides were found that segregated concordantly with the human X chromosome. The regions of the gel that contain these five polypeptides are indicated by the lettered boxes. In figure 4, each of these five lettered regions is enlarged and compared in gels from hybrid clones with and without the human X chromosome. Each of the five polypeptides indicated by the arrows represents a putative human X-linked gene product.

To establish that these five polypeptides are human and not rodent gene products, radioactive human fibroblast extracts were mixed with radioactive extracts from hybrids containing the putative human X-linked proteins. Autoradiographs of these mixing gels were then compared with autoradiographs of gels containing only human fibroblast polypeptides. Such a comparison for polypeptide D, illustrated in figure 5, reveals that this polypeptide comigrates with a human fibroblast protein, but not with any of the polypeptides present in the rodent parental cell. Similar comparisons for polypeptides A, B, C, and E revealed that each of these putative human X-linked polypeptides comigrates with a protein present in the human fibroblast extract, but not with any of the proteins present in rodent parental cell extracts. Although peptides B and C each comigrate with a human fibroblast protein, the migration of B and C is also very close to that of several rodent proteins that are present in low amounts in the rodent parental cells and hybrid clones (fig. 4). However, an analysis of numerous gels has consistently shown that B and C migrate slightly differently than these rodent proteins. Nevertheless, we are clearly at the limits of resolution of the system when we identify B and C as human polypeptides rather than as rodent proteins whose synthesis is increased because of the presence of a human chromosome.

While the human polypeptides A, B, C, D, and E are all present in each of the mouse-human and hamster-human hybrid clones that contain a human X chromosome, these polypeptides are absent in all hybrids without a human X. Since no human autosome consistently segregates with the human X chromosome or is consistently present in association with the human X chromosome in the hybrid clones used in this study (table 1), these results strongly suggest that polypeptides A, B, C, D, and E are human X-linked gene products.

*Characterization of the Five Human X-Linked Polypeptides*

The molecular properties of the five human X-linked polypeptides identified by 2-D gel electrophoresis are indicated in table 2. Polypeptide pX-56 has a mol. wt. of 56,000 daltons and an apparent isoelectric point of pH 6.8. These molecular properties are similar to those reported for the subunit of the human X-linked


A
1
2
3
6
7
8
9
11
12
14
19
B
3/X
3/5
4/4
5/5
4/15
9/9
10/15
10/16
14/15
5/?
9/17
13/13
17/18
C
D
HUMAN
X

enzyme: G6PD-B [7]. To determine if polypeptide pX-56 comigrates with the human G6PD-B subunit, purified nonradioactive human erythrocyte G6PD-B was mixed with a radioactive extract from a hybrid cell containing pX-56, and this mixture was analyzed by 2-D gel electrophoresis. The gel was first stained with Coomassie Blue to localize the purified G6PD subunit, and then exposed to X-ray film to localize pX-56. When the gel was stained for protein, the purified G6PD subunit was found to migrate as three polypeptide spots, all with a mol. wt. of 56,000 daltons but varying in charge. These results are consistent with a previous report [7]. Since only a small amount of radioactive protein was applied to the gel, the majority of the radioactive polypeptides were not present in sufficient quantity to be detected by the protein stain, and as a result, they did not obscure the identification of the purified G6PD subunit. When an autoradiograph of this gel was superimposed over the stained gel, pX-56 was found to comigrate with the most basic of the G6PD-B polypeptide spots (fig. 6). These results strongly suggest that pX-56 is the polypeptide subunit of the human X-linked enzyme G6PD.

The polypeptide subunit of another human X-linked enzyme, HPRT, is reported to have a mol. wt. of 26,000 daltons and an isoelectric point of approximately pH 6 [16]. Such a polypeptide should have been detected using our 2-D gel system. To determine if any of the polypeptides listed in table 2 comigrate with the human HPRT subunit, we mixed purified nonradioactive human erythrocyte HPRT with radioactive extracts from hybrids containing a human X chromosome, as well as with radioactive extracts containing human, mouse, or hamster proteins alone, and performed an analysis similar to that described for G6PD. When the gels were stained for protein, the purified erythrocyte HPRT subunit was found to migrate as one major and one minor polypeptide spot, each with a mol. wt. of 26,000 daltons. The most basic of these spots has been shown to correspond to the HPRT subunit found in human fibroblasts [17]. When an autoradiograph of a gel containing radioactive human fibroblast proteins was superimposed over the protein stained gel, the most basic HPRT spot was found to comigrate with a radioactive human fibroblast polypeptide that did not correspond to any of the five human polypeptides listed in table 2. However, there was also a radioactive polypeptide in extracts from the parental cells that comigrated with the most basic human HPRT spot (fig. 7). In addition, extracts from the mouse parental cells contained a polypeptide that comigrated with the purified human HPRT. This comigration of human HPRT

FIG. 1.—Composite karyotype of mouse-human hybrid clones A9498-1 HAT (*top line*) and A9498-1 AZA (*bottom line*). Chromosomes placed in *section A* are apparently structurally normal mouse chromosomes identified by their respective nos. according to standard nomenclature. Chromosomes placed in *section B* are Robertsonian fusion products of recognizable derivation. The interpretation of their constituents is indicated by nos. below chromosomes with first no. designating the lower arm and no. after *dash* designating the upper arm. These Robertsonian fusion chromosomes are characteristic of the mouse A9 cell line. Chromosomes placed in *section C* are rearranged chromosomes, some of them metacentric, most of them telocentric, of undetermined origin. The respective chromosomes in HAT (*top*) and AZA (*bottom*) derivatives are mounted *above each other*. *Section D* shows the normal human X chromosome present in the HAT clone and absent in the AZA clone. There were no other recognizable human chromosomes in these particular metaphase spreads, although human chromosomes 12 and 21 were represented in approximately 50% of cells.

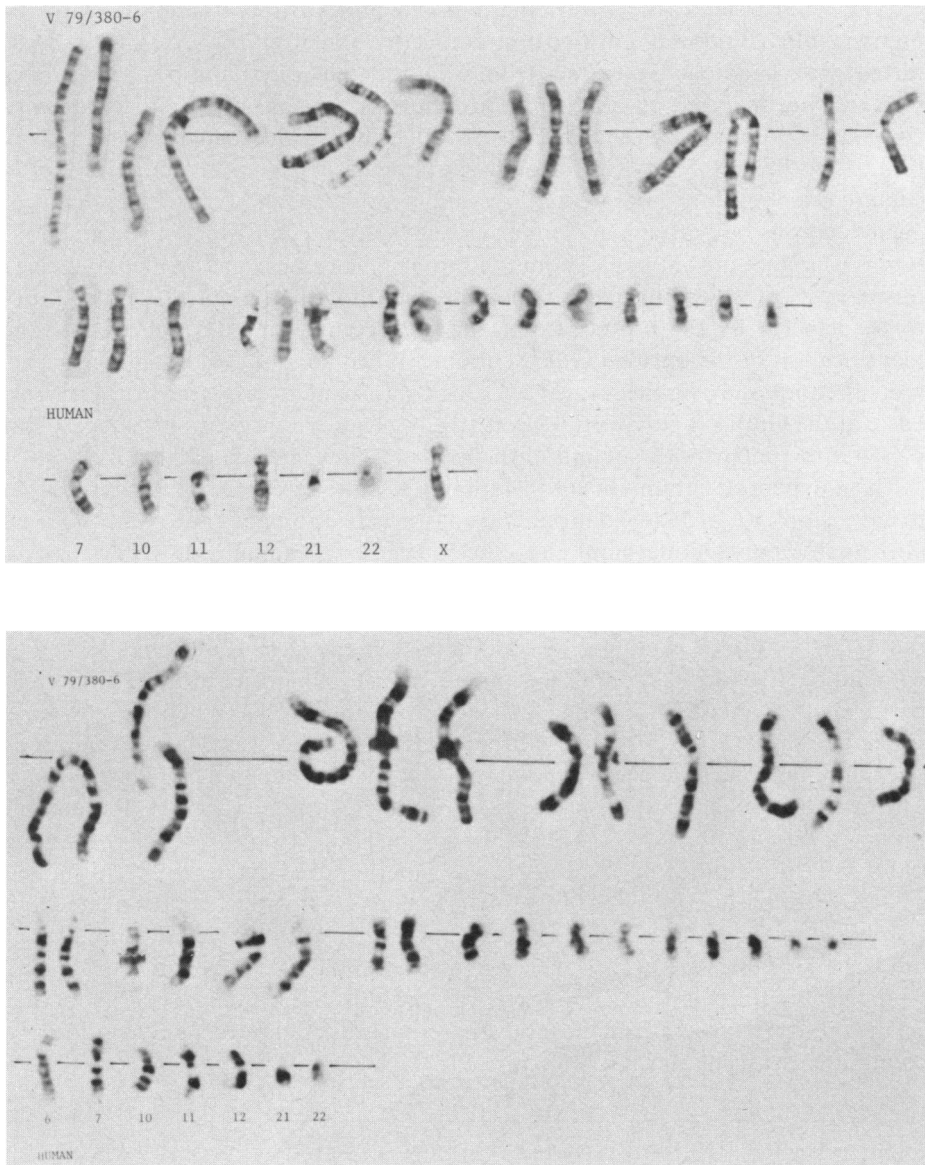



FIG. 2.—*A* (*top*), Chinese hamster-human hybrid clone designated CH3-1 Hat. Chinese hamster (cell line V79/380-6) chromosomes are arranged in *upper two rows* and human chromosomes in *bottom row*. All human chromosomes are structurally intact. *B* (*bottom*), Chinese hamster-human hybrid clone CH3-1 AZA, derived from clone CH3-1 HAT by counterselection in azaguanine. Chinese hamster V79/380-6 chromosomes are arranged in *upper two rows* and human chromosomes in *bottom row*. A secondary rearrangement involving Chinese hamster chromosome 1 was identical in both clones. The AZA clone has lost the human X chromosome but has retained all the human autosomes present previously.

TABLE 1

HUMAN CHROMOSOMES PRESENT IN RODENT-HUMAN HYBRID CLONES

| | HUMAN CHROMOSOMES* | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X | Xp+† |
| Mouse-Human clones: | | | | | | | | | | | | | | | | | | | | | | | | |
| A9498-1 HAT | | | | | | | | | | | | + | | | | | | | | | + | | + | |
| A9498-1 AZA | | | | | | | | | | | | + | | | | | | | | | + | | | |
| Hamster-Human clones: | | | | | | | | | | | | | | | | | | | | | | | | |
| CH3-1 HAT | | | | | | + | + | | | + | + | + | | | | | | | | | + | + | + | |
| CH3-1 AZA | | | | | | + | + | | | + | + | + | | | | | | | | | + | + | | |
| XIII-7A HAT | | | | | + | | + | + | + | | | | + | | + | | + | + | | + | | + | | + |
| XIII-7A AZA | | | | | + | | + | + | + | | | | + | | + | | + | + | | + | | + | | |
| XIII-4D HAT | | | + | + | | + | | + | | | + | | | | + | + | | + | | + | + | | | + |
| XIII-4D AZA | | | | | | + | | | | | | | | | + | | | | | | + | | | |

* Hybrids were scored "+" if chromosome was present in more than 15% of cells.
† der(X)t(X;14)(p22;q21).

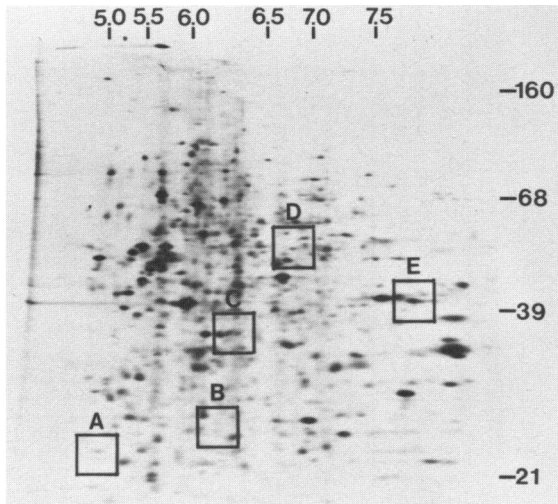



FIG. 3.—Complete 2-D autoradiograph of polypeptides from a mouse-human hybrid clone containing a human X chromosome. Regions of the gel containing polypeptides present in hybrids with a human X chromosome but absent from clones without a human X are indicated by *lettered boxes*. Here and in figures 4–8, the basic side of the gel is on the *right*. Apparent isoelectric points are noted along the *top* of the gel. Separation in the vertical direction is according to molecular weight, indicated along the *side* of the gel in daltons $\times 10^{-3}$.

with a rodent protein in extracts from both the hamster and mouse parental cells explains why HPRT was not identified as one of the human X-linked polypeptides in the rodent-human hybrids.

Polypeptides pX-24, pX-27, pX-37, and pX-40 have molecular properties that are unlike those of known human X-linked proteins, and may represent products of human X-linked loci that have not been previously described. To determine the subcellular localization of each of these polypeptides, a radioactive cell extract containing all five of the human proteins listed in table 2 was fractionated into a crude membrane pellet and a crude cytoplasmic supernatant, as described in MATERIALS AND METHODS. Approximately 50% of the total radioactivity was present in each fraction. Equivalent amounts of trichloroacetic acid-precipitable radioactivity from the crude membrane pellet, the crude cytoplasmic supernatant, and the unfractionated cell extract were analyzed by 2-D gel electrophoresis to determine the relative amounts of each of the five polypeptides in the individual subcellular fractions. As shown in figure 8, while the crude membrane fraction is greatly enriched for pX-24 as compared to the unfractionated cell extract, this same membrane fraction is depleted for pX-40. The results of such an analysis for all five of the X-linked polypeptides listed in table 2 indicate that pX-27, pX-37, pX-40, and pX-56 are soluble proteins, while pX-24 is membrane bound. Trypsinization of the cells prior to the preparation of extracts did not affect any of these five proteins, either quantitatively or qualitatively.

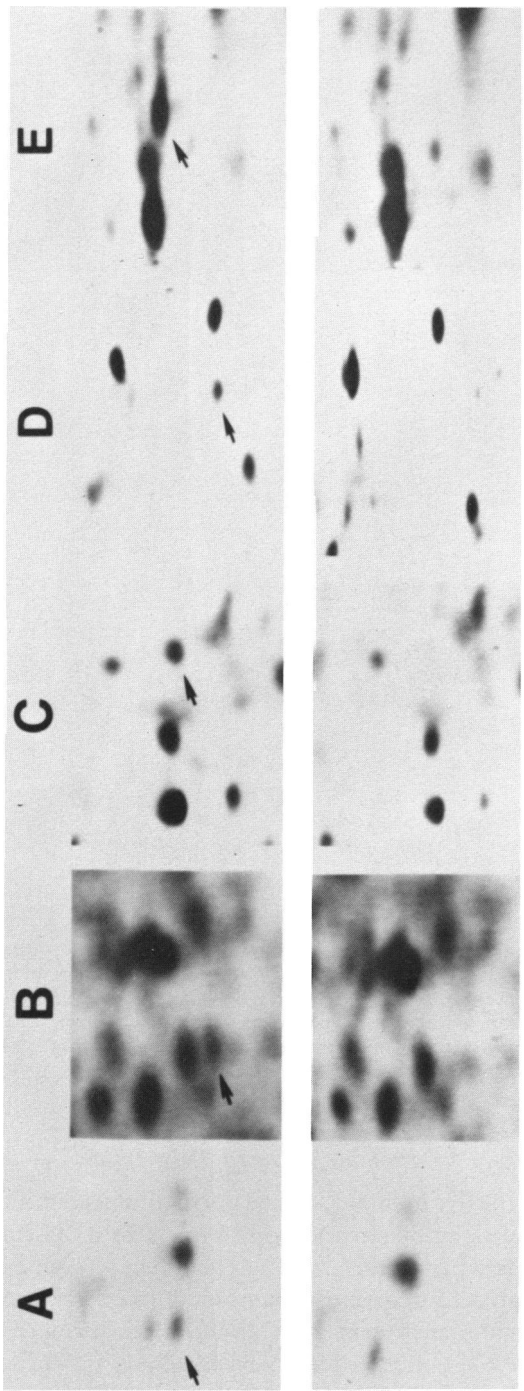



FIG. 4.—Polypeptides expressed only in presence of a human X chromosome. Each of the *five lettered regions* in figure 3 is enlarged and compared in gels from hybrid clones with (*top panels*) and without (*bottom panels*) a human X chromosome. In each *panel*, *arrow* indicates location of a polypeptide that is expressed only in extracts from hybrid clones that contain a human X chromosome.

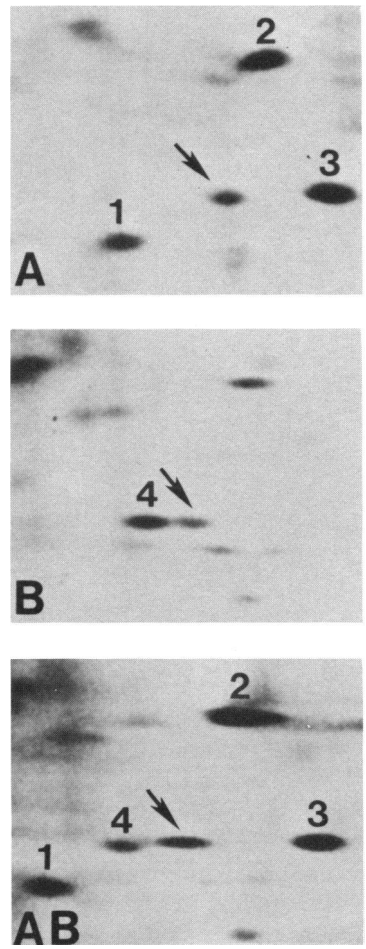



FIG. 5.—Comigration of polypeptide D with a human fibroblast protein. *A*, Autoradiograph of a radioactive extract from a hamster-human hybrid containing a human X chromosome. Polypeptide D, defined in figure 4, is indicated by *arrow*. *B*, Autoradiograph of a radioactive human fibroblast extract. The human polypeptide that comigrates with polypeptide D is indicated by *arrow*. *AB*, Autoradiograph of a mixture of the extracts in *A* and *B*, demonstrating comigration of polypeptide D with a human fibroblast protein.

### *Regional Mapping of the Five Human X-Linked Polypeptides*

Regional mapping of the five human X-linked polypeptides identified on 2-D gels was performed using mouse-human hybrid clone A9X. Alkaline Giemsa staining of metaphase spreads prepared from this clone revealed that a fragment of a human chromosome, translocated to a mouse chromosome, was the only identifiable human chromosomal material present in this hybrid. Since clone A9X contains human G6PD and HPRT but not human 3-phosphoglycerate kinase (PGK) (E.C.2.7.2.3) as determined by Cellogel electrophoresis, the translocated human

chromosome fragment in this clone appears to be the distal long arm of the human X chromosome. When radioactive extracts of clone A9X were analyzed by 2-D gel electrophoresis, polypeptides pX-24 and pX-56 were present, while pX-27, pX-37, and pX-40 were absent. These results suggest that polypeptides pX-24 and pX-56 map to the distal long arm of the human X chromosome, while polypeptides pX-27, pX-37, and pX-40 map to the proximal long arm or to the short arm of the human X.

## DISCUSSION

We have used the technique of 2-D gel electrophoresis to analyze radioactive extracts from rodent-human somatic cell hybrids segregating human chromosomes, and have found five polypeptides in hybrid cells containing a human X chromosome that are absent in hybrid cells without a human X. The human origin of these five polypeptides has been established by their comigration with proteins in human fibroblast extracts, and by their failure to comigrate with proteins in mouse or hamster cell extracts. The identical migration of each of these five polypeptides in extracts from both mouse-human and hamster-human hybrids, and the finding that the presence of these five polypeptides is not associated with the simultaneous loss of any rodent protein in the hybrid extracts, further suggests that these five polypeptides are human in origin, and not rodent proteins that have been modified by a human X-linked gene product. We can identify these five polypeptides as gene products of the human X chromosome, since the human X chromosome is both necessary and sufficient for the expression of all five polypeptides in the hybrid clones used in this study. These five human X-linked polypeptides have been designated pX-24, pX-27, pX-37, pX-40, and pX-56.

The comigration of pX-56 with purified human erythrocyte G6PD-B provides strong evidence that pX-56 is the subunit protein of the human X-linked enzyme G6PD. The observation that pX-56 is a soluble protein and the demonstration that the locus coding for this polypeptide maps to the distal long arm of the human X chromosome are findings consistent with the identification of pX-56 as human G6PD. While purified human erythrocyte G6PD migrates as three spots on 2-D gels, only one of these spots appears to comigrate with a human fibroblast polypep-

TABLE 2

MOLECULAR PROPERTIES OF THE HUMAN X-LINKED POLYPEPTIDES IDENTIFIED BY TWO-DIMENSIONAL ELECTROPHORESIS

| | HUMAN POLYPEPTIDE | | | | |
|---|---|---|---|---|---|
| | A | B | C | D | E |
| Molecular weight ($\times 10^{-3}$) | 24 | 27 | 37 | 56 | 40 |
| Apparent isoelectric point | 4.9 | 6.2 | 6.3 | 6.8 | $> 7.6$ |
| Membrane-bound | + | − | − | − | − |
| Purified human protein which comigrates with the X-linked polypeptide | ... | ... | ... | G6PD | ... |
| Nomenclature | pX − 24 | pX − 27 | pX − 37 | pX − 56 | pX − 40 |

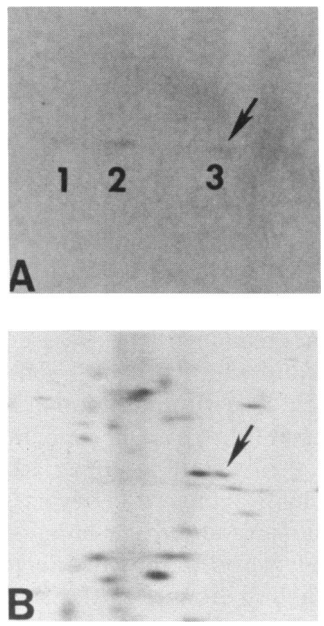



FIG. 6.—Comigration of polypeptide D (pX-56) with human G6PD-B. Purified nonradioactive human erythrocyte G6PD-B was mixed with a radioactive extract from a hamster × human hybrid clone containing pX-56, and this mixture was analyzed by 2-D gel electrophoresis. *A* shows the region of the Coomassie Blue-stained gel that contains the three erythrocyte G6PD-B polypeptides (see text). *Arrow* in *A* indicates the most basic of these polypeptides. *B* is an autoradiograph of that region of the gel shown in *A*. Arrow in *B* indicates pX-56. Since *arrows* in *A* and in *B* point to the same position on the stained gel and on the autoradiograph, this figure demonstrates the comigration of pX-56 with the most basic polypeptide subunit of human erythrocyte G6PD-B.

tide. Previous studies comparing erythrocytic and fibroblastic forms of other human enzymes have revealed similar findings, and suggest that the posttranslational modification of many human enzymes in erythrocytes is tissue specific [18].

Polypeptides pX-24, pX-27, pX-37, and pX-40 have molecular properties that are unlike those of known human X-linked gene products. However, the finding that pX-24 is membrane bound and maps to the distal long arm of the X chromosome suggests that this polypeptide may be related to the serologically defined human X-linked cell surface antigen(s) that have recently been described and that also map to the distal long arm of the human X [19–21]. It will be important to determine if pX-24 is a cell surface protein and if it can be immunoprecipitated by any of the serologic reagents that are presently used to define human X-linked surface antigens.

The loci coding for polypeptides pX-27, pX-37, and pX-40 appear to map proximal to the human HPRT locus, but it is not yet known if they are located on the short arm or on the proximal long arm of the human X chromosome. In view of the recent finding that certain loci that map to the short arm of the human X chromosome escape X-inactivation [22, 23], it will be important to complete the regional

mapping of pX-27, pX-37, and pX-40 and to determine if these polypeptides show quantitative differences in XX vs. XY human cells.

Although the human X-linked enzymes PGK, $\alpha$-galactosidase ($\alpha$-gal) (E.C.3.2.1.22) and HPRT are present in extracts of the rodent-human hybrid cells containing a human X chromosome, as determined by Cellogel electrophoresis, the polypeptide subunits of these enzymes were not identified as human X-linked gene products here. This is not surprising, however, since the human HPRT polypeptide could not be distinguished from rodent proteins on the 2-D gels, and since the polypeptide subunits of PGK and $\alpha$-gal have molecular properties that would have excluded them from the gels [24, 25].

Under the conditions used in this study, we should be able to distinguish 180 human polypeptides from rodent proteins in extracts of hamster-human and mouse-human hybrid cells. If we assume that these 180 polypeptides are coded for by loci

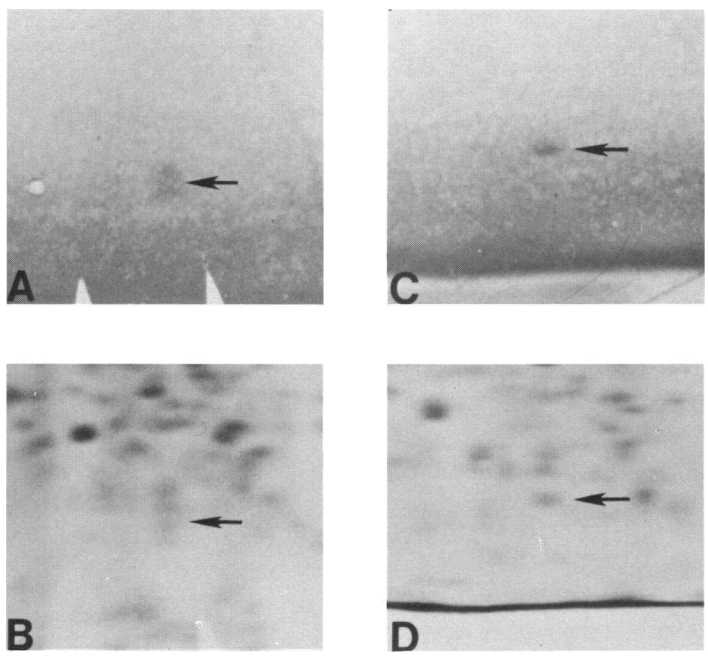



FIG. 7.—Comigration of human HPRT with proteins in both human and hamster cell extracts. Purified nonradioactive human HPRT was mixed with a radioactive human fibroblast extract or a radioactive hamster cell extract, and these mixtures were analyzed by 2-D gel electrophoresis. *A* shows the region of the Coomassie Blue-stained gel that contains nonradioactive human HPRT, indicated by *arrow*, and radioactive human fibroblast proteins. *B* is an autoradiograph of *A*. *Arrows* in *A* and *B* point to the same position on the stained gel and the autoradiograph, demonstrating comigration of human HPRT with a human fibroblast polypeptide. *C* shows the region of the Coomassie Blue-stained gel that contains nonradioactive human HPRT, indicated by *arrow*, and radioactive Chinese hamster proteins. *D* is an autoradiograph of *C*. *Arrows* in *C* and *D* point to the same position on the stained gel and the autoradiograph, demonstrating the comigration of human HPRT with a hamster protein. In *A* and *C*, only the major, most basic human erythrocyte HPRT polypeptide is present in sufficient quantity to be visualized by protein staining.

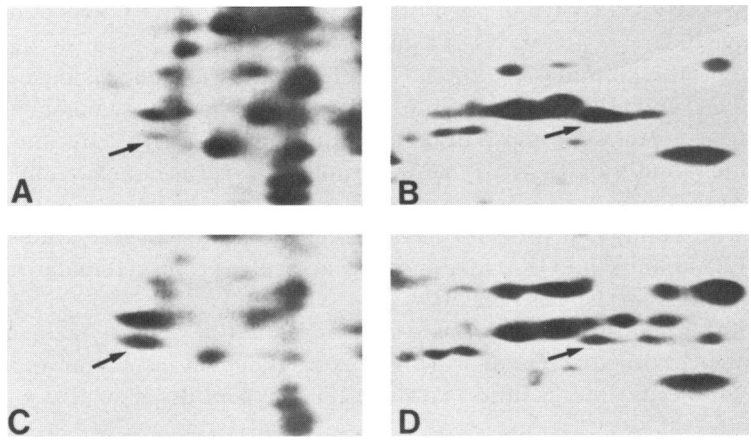



FIG. 8.—Subcellular localization of human X-linked polypeptides. *A* and *B* are regions of a gel from an unfractionated cell extract of hybrid clone A9498-1 HAT that contains a human X chromosome. *C* and *D* are the analogous regions of a gel from a crude membrane fraction of clone A9498-1 HAT. Equal amounts of radioactive protein were applied to each gel, and both autoradiographs were exposed for the same length of time. Polypeptide pX-24, indicated by *arrows* in *A* and *C*, is greatly enriched in the crude membrane fraction compared to the unfractionated cell extract, while polypeptide pX-40, indicated by *arrows* in *B* and *D*, is depleted in the crude membrane fraction.

that are randomly distributed throughout the human genome, that the number of polypeptides coded for by a chromosome is proportional to the amount of DNA in that chromosome, and that the human X chromosome accounts for about 5% of the DNA in the haploid human genome [26], approximately nine of the 180 human proteins that are distinguishable from rodent proteins should be gene products of the human X chromosome. The five such polypeptides that we identified are close to the theoretical expectation.

The electrophoretic conditions used here allow us to identify approximately 400 polypeptides from crude human fibroblast extracts, ranging in size from 20,000 to 200,000 daltons with isoelectric points between pH 4.7 and pH 7.5. Polypeptides comprising as little as 0.1% of the total cell protein were easily detected. There is abundant evidence that polypeptides differing by a single charge can be clearly resolved under these conditions [27–29]. Since it has been estimated that approximately 80%–90% of the human enzymes analyzed by starch gel or cellulose acetate electrophoresis can be distinguished from the homologous rodent enzymes [30], it was quite surprising that only 45% of the human polypeptides identified on the 2-D gels could be distinguished from either mouse or hamster polypeptides, and that only 15% of these human polypeptides could be distinguished from the rodent proteins in both mouse and hamster cell extracts. In a recent study comparing several hundred polypeptides from four human diploid fibroblast lines by high resolution 2-D gel electrophoresis, McConkey et al. [31] found an average heterozygosity of less than 1% for changes involving charged amino acids, which is much lower than the predicted heterozygosity based on an extrapolation of human enzyme survey data. There are several hypotheses that may explain why the proteins

identified by 2-D gel electrophoresis show fewer differences among species and less polymorphism within species than expected on the basis of data from enzyme surveys [31]. Whatever the explanation, the inability to distinguish a large proportion of human polypeptides from rodent proteins on 2-D gels obviously limits the number of human proteins that can be mapped using this technique.

Another feature that limits the usefulness of 2-D gel electrophoresis for gene mapping involves the analysis of the gels themselves. Finding a few spots unique to one 2-D pattern among hundreds of spots common to several patterns is a formidable task. The visual side by side comparison that was used to analyze the gels in this study is very laborious. However, a recently described method that permits the identification of [$^3$H]-labeled polypeptides on gels that contain both [$^{14}$C]- and [$^3$H]-labeled proteins should greatly facilitate 2-D gel comparison [32].

In spite of its limitations, 2-D gel electrophoresis remains a powerful tool that can be used to study hundreds of primary gene products not previously amenable to genetic analysis. 2-D gel analysis of extracts from a panel of somatic cell hybrid clones, informative for a particular chromosome, provides a general method for identifying those polypeptides in crude cell extracts that are coded for by that chromosome. Such an approach is not dependent on the biologic function of the polypeptides being analyzed, and should be particularly useful in future genetic studies of structural proteins.

## ACKNOWLEDGMENTS

We thank Drs. Akira Yoshida and Roberta Palmour for providing preparations of purified human erythrocyte enzymes.

## REFERENCES

1. Donald LJ, Hamerton JL: A summary of the human gene map, 1973–1977. *Cytogenet Cell Genet* 22:5–11, 1978
2. Nichols EA, Ruddle FH: A review of enzyme polymorphism, linkage and electrophoretic conditions for mouse and somatic cell hybrids in starch gels. *J Histochem Cytochem* 21:1066–1081, 1973
3. van Someren H, Beijersbergen van Henegouwen H, Los W, et al.: Enzyme electrophoresis on cellulose acetate gel. *Humangenetik* 25:189–201, 1974
4. Nichols EA, Ruddle FH: Comparative sensitivities of electrophoretic assays for human enzymes. *Biochem Genet* 17:127–132, 1979
5. O'Farrell PH, O'Farrell PZ: Two-dimensional polyacrylamide gel electrophoretic fractionation. *Methods Cell Biol* 16:407–420, 1978
6. O'Farrell PH: High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250:4007–4021, 1975
7. Edwards JL, Anderson NG, Nance SL, Anderson NL: Red cell proteins I. Two-dimensional mapping of human erythrocyte lysate proteins. *Blood* 53:1121–1132, 1979
8. Elliott RW: Use of two-dimensional electrophoresis to identify and map new mouse genes. *Genetics* 91:295–308, 1979
9. McConkey EH: Identification of human gene products from hybrid cells: a new approach. *Somatic Cell Genet* 6:139–147, 1980
10. Littlefield JW: Selection of hybrids from matings of fibroblasts in vitro and their presumed recombinants. *Science* 145:709–710, 1964
11. Francke U, Busby N, Shaw D, Hansen S, Brown MG: Intra-chromosomal gene mapping in man: assignment of nucleoside phosphorylase to region 14 cen→14q21 by interspecific

hybridization of cells with a t(X;14)(p22;q21) translocation. *Somatic Cell Genet* 2:27–40, 1976

12. DAVIDSON RL, O'MALLEY KA, WHEELER TB: Polyethylene glycol-induced mammalian cell hybridization: effect of polyethylene glycol molecular weight and concentration. *Somatic Cell Genet* 2:271–280, 1976
13. FRANCKE U, DENNEY RM, RUDDLE RH: Intra-chromosomal gene mapping in man: the gene for tryptophanyl-tRNA synthetase maps in region q21→qter of chromosome 14. *Somatic Cell Genet* 3:381–389, 1977
14. OLIVER N, FRANCKE U, PELLEGRINO MA: Regional assignment of genes for mannose phosphate isomerase, pyruvate kinase-3, and β2-microglobulin expression on human chromosome 15 by hybridization of cells from a t(15;22)(q14;q13.3) translocation carrier. *Cytogenet Cell Genet* 22:506–510, 1978
15. ALHADEFF B, VELIVASKIS M, SINISCALCO M: Simultaneous identification of chromatid replication and of human chromosomes in metaphases of man-mouse somatic cell hybrids. *Cytogenet Cell Genet* 19:236–239, 1977
16. O'FARRELL PZ, GOODMAN HM, O'FARRELL PH: High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* 12:1133–1142, 1977
17. GHANGAS GS, MILMAN G: Hypoxanthine phosphoribosyltransferase: two-dimensional gels from normal and Lesch-Nyhan hemolyzates. *Science* 196:1119–1120, 1977
18. ZANNIS VI, GUDAS LJ, MARTIN DW: Characterization of the subunit composition of HGPRTase from human erythrocytes and cultured fibroblasts. *Biochem Genet* 18:1–9, 1980
19. BUCK EW, BODMER WF: Serological identification of an X-linked human cell surface antigen, SA-X. *Cytogenet Cell Genet* 16:376–377, 1976
20. DORMAN BP, SHIMUZU N, RUDDLE FH: A cell surface antigen linked to the human X chromosome and present on fibroblasts. *Cytogenet Cell Genet* 22:243–244, 1978
21. SCHWAB R, SINISCALCO M: Confirmative evidence for a human X-linked surface antigen and its tentative regional assignment. *Cytogenet Cell Genet* 22:538–540, 1978
22. SHAPIRO LJ, MOHANDAS T, WEISS R, ROMEO G: Non-inactivation of an X-chromosome locus in man. *Science* 204:1224–1226, 1979
23. MOHANDAS T, SHAPIRO LJ, SPARKES RS, SPARKES MC: Regional assignment of the steroid sulfatase-X-linked ichthyosis locus: implications for a non-inactivated region on the short arm of the human X chromosome. *Proc Natl Acad Sci USA* 76:5770–5783, 1979
24. MAPES CA, SUELTER CH, SWEELEY CC: Isolation and characterization of ceramide trihexosidases (form A) from human plasma. *J Biol Chem* 248:2471–2479, 1973
25. ALI M, BROWNSTONE YS: A study of phosphoglycerate kinase in human erythrocytes I. Enzyme isolation, purification and assay. *Biochim Biophys Acta* 445:74–88, 1976
26. BOSMAN FT, VAN DER PLOEG M, VAN DUIJN P, SCHABERG A: Photometric determination of the DNA distribution in the 24 human chromosomes. *Exp Cell Res* 105:301–311, 1977
27. STEINBERG RA, O'FARRELL PH, FRIEDRICH U, COFFINO P: Mutations causing charge alterations in regulatory subunits of the c-AMP-dependent protein kinase of cultured S49 lymphoma cells. *Cell* 10:381–391, 1977
28. ANDERSON L, ANDERSON NG: High resolution two-dimensional electrophoresis of human plasma proteins. *Proc Natl Acad Sci USA* 74:5421–5425, 1977
29. PARKER J, POLLARD JW, FREISEN JD, STANNERS CP: Stuttering: high level mistranslation in animal and bacterial cells. *Proc Natl Acad Sci USA* 75:1091–1095, 1978
30. MEERA KHAN P: Enzyme electrophoresis on cellulose acetate gel: zymogram patterns in man-mouse and man-Chinese hamster somatic cell hybrids. *Arch Biochem Biophys* 145:470–483, 1971
31. MCCONKEY EH, TAYLOR BJ, PHAN D: Human heterozygosity: a new estimate. *Proc Natl Acad Sci USA* 76:6500–6504, 1979
32. MCCONKEY EH: Double-label autoradiography for comparison of complex protein mixtures after gel electrophoresis. *Anal Biochem* 96:39–44, 1979