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Enzyme Electrophoresis on Cellulose Acetate Gel: Zymogram Patterns in Man–Mouse and Man–Chinese Hamster Somatic Cell Hybrids[1]

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Because of the occurrence of preferential loss of human chromosomes and the existance of built-in enzyme markers, the man–mouse and man–Chinese hamster somatic cell hybrid systems have become important tools for genetic analysis of man. Cellulose acetate gel (Cellogel) was found to have certain additional advantages over the other supporting media for zymogram analysis. Therefore, techniques, developed during the course of a series of investigations on somatic cell hybrids in our laboratory, are described for the electrophoretic characterization of glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate-dependant isocitrate dehydrogenase, lactate dehydrogenase, nicotinamide–adenine dinucleotide-dependant malate dehydrogenase, 6-phosphogluconate dehydrogenase, indophenole oxidase (1), hypoxanthine–guanine phosphoribosyl transferase, phosphoglucomutase, and phosphoglycerate kinase[2] in the man–mouse and man–Chinese hamster somatic cell hybrid systems, on Cellogel. The results are briefly discussed.

It is an established fact that a single amino acid substitution in a polypeptide chain may change the electrophoretic mobility of the constituted protein. Enzyme protein was shown to be no exception to this effect (2). This is obviously due to a change in the net charge of the protein molecule. This happens usually when the replacing amino acid carries a charge different from that of the replaced amino acid. This is presumably a reflection of the substitution of the corresponding base-pair in that part of the DNA molecule which determines the structure of the protein molecule, *i.e.*, a mutation in the structural gene.

The process of evolution of the species can be comprehended as an accumulation of adaptive mutations. Therefore, the evolutionary divergence between different species would naturally result in the occurrence of enormous interspecific variation with respect to the nucleotide sequences at their homologous genetic loci. Thus, the interspecific somatic cell hybrids such as man–mouse (3) or man–Chinese hamster (4) would present a huge catalogue of built-in genetic markers.

The number of available genetic markers for the study of these hybrids is, therefore, practically limited by the number of proteins or enzymes for which proper assay systems are available to distinguish the human type from the type of the other partner in the hybrid cell. The most widely used assay procedure has been the zymogram method.

The preferential loss of human chromosomes from the man–mouse and man–Chinese hamster somatic cell hybrids would lead to the formation of a spectrum of hybrid cells with varying numbers and combinations of human chromosomes, in addi-

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[2] The IUB Enzyme Commission numbers and the systematic names corresponding to this trivial nomenclature of these enzymes appear in Table III.

tion to the complete set of mouse or Chinese hamster chromosomes, as the case might be.

The foregoing features have made these hybrid systems ideal tools for human genetic analysis. The rationale for such a study is simple and straightforward. Linked genes are either retained or lost together. They do not dissociate in the absence of chromosomal rearrangement. Thus, consistent phenotypic association suggests linkage and nonassociation means nonlinkage, provided the phenotypic expressions of the genes involved are not subject to regulation. The strategy of electrophoretic analysis of enzymes in the man–mouse somatic cell hybrids for the genetic analysis of man has been thoroughly discussed and a handful of studies are on record to substantiate the importance of zymogram approach to genetic studies on somatic cell hybrids (5).

Many types of supporting media have been used for enzyme electrophoresis ever since Hunter and Markert (6) developed a method for demonstrating enzymes on starch gel by zone electrophoresis followed by histochemical staining.

Cellulose acetate gel (Cellogel from Chemetron, via G. Modena, 24, Milan, Italy) is one such medium available in the ready-to-use form. Neither cooling nor refrigeration is necessary during electrophoresis. Relatively large numbers of samples can be assayed economically in a short time. Separation of most proteins is rapid and clear. The aliquots required for assays, the amounts of various reagents used for staining, and the incubation period during staining are minimal. The Cellogel technique has been routinely used in this laboratory for screening human populations for the red cell enzyme polymorphisms (7–12).

The purpose of the present paper is to describe the techniques of Cellogel electrophoresis for certain enzymes employed by us in studying the man–mouse and man–Chinese hamster somatic cell hybrids (4, 13–16).

MATERIALS AND METHODS

The man–mouse and man–Chinese hamster somatic cell hybrids and their parent cell lines used in these studies were kindly provided by Drs. O. J. Miller, S. Shin, A. Westerveld, K.-H. Grzeschik, and B. Deys and they were described in detail elsewhere (4, 13, 15). Methods are described here for characterizing the electrophoretic behavior of G6PD[3], NADP–IDH, LDH, NAD–MDH, 6-PGD, IPO, HGPRT, PGM, and PGK in the human (Ms2, Ms62, and Ms64), mouse (L_{929}, A_9, and B_{82}) and Chinese hamster (DON) cells, and in man–mouse (A_9/RBC and A_9/ADC) and/or man–Chinese hamster (a_3/Ms2, a_{23}/Ms2, and Wg3/Lymph) somatic cell hybrids. Cellogel in sheets (16 × 17 cm) of 0.5-mm thickness is used as the supporting medium of electrophoresis (9). This thickness of the gel is critical for ensuring a good separation and sharp appearance of the enzyme bands.

Preparation of cell lysates. About 5×10^6 cells, trypsinized and suspended in the culture medium, are washed thrice with isotonic saline. They are resuspended in 0.15 ml of "lysis" buffer (see below) and sonicated for 10 sec in an ice bath. The energy level is set at 7, when the Sonocone sonicator (of Powertron Division, Giannini Control Corp., Plainview, N. Y.) is used. The sonicate is mixed thoroughly with 0.1 ml of carbon tetrachloride and centrifuged at 27,000*g* for 30 min at 4°. A clear supernatant fluid gives good results, up to 1 week if stored at 4°. Storage at −20° preserves the enzymes longer, but may result in distorted patterns of G6PD on electrophoresis. In our experience the extracts of freshly trypsinized, unfrozen cells gave the best results.

Lysis buffer. This comprises 5×10^{-3} M phosphate buffer, pH 6.4, containing 1×10^{-3} M Na_2EDTA, 1×10^{-3} M β-mercaptoethanol, 1×10^{-4} M di-isopropylfluorophosphate (DFP), and 2×10^{-5} M nicotinamide adenine dinucleotide phosphate (NADP). These constituents are found to be protective to the enzymes, especially when the assays cannot be performed soon after sonication.

Electrophoresis. The electrophoretic buffer systems for these enzymes are described in Table I. Table II includes details of all the reagents used in different reaction mixtures. The enzymes are

[3] Abbreviations: G6PD, glucose-6-phosphate dehydrogenase; NADP–IDH, nicotinamide adenine dinucleotide phosphate-dependent isocitrate dehydrogenase; LHD, lactate dehydrogenase; NAD–MDH, nicotinamide adenine dinucleotide-dependent malate dehydrogenase; 6-PGD, 6-phosphogluconate dehydrogenase; IPO, indophenol oxidase (1); HGPRT, hypoxanthine–guanine phosphoribosyl transferase; PGM, phosphoglucomutase; PGK, phosphoglycerate kinase; TK, thymidine kinase.

listed in Table III with particulars of their electrophoresis and staining.

The Cellogel stored in 50% methanol is blotted between two sheets of fine-grained filter paper and soaked in appropriate electrophoretic buffer for about 10 min. The gel is blotted and soaked in fresh buffer twice more and left under the same buffer for use. Care has been exercised not to allow the formation of white patches in the gel, due to excessive drying, throughout the entire procedure.

Shandon Universal Electrophoresis tank (MK II or U77) is used with an in-between shoulder-gap of 9 cm. The buffer-impregnated gel is blotted and positioned in the tank with its penetrable surface facing upward and its free ends hanging into the buffer in the end compartments. Keeping the amperage constant, the initial voltage is set to 200 V and the system is equilibrated for 10 min.

The current is broken and a 2- to 3-μl aliquot of each sample is applied with a 2-μl Pleuger micropipette at about 5 mm (but 20 mm for LDH) away from and parallel to the cathodic shoulder piece as a straight streak. Ten to twenty samples can be assayed on a standard sheet (16 × 17 cm) depending upon the type of enzyme and purpose of electrophoresis. On complete adsorption of the samples applied on the gel, the current is made again and the starting time is noted. The buffer system, the duration of electrophoresis, and the composition of specific reaction mixture for each enzyme are described separately in Table III.

A required amount of appropriate reaction mixture is freshly prepared at the end of each run. The current is broken; the gel is marked, and the hanging ends are cut off. The porous side of the gel is repeatedly brought into contact with the reaction mixture spread on a glass plate for about 30 sec for all the enzymes but 6PGD (see Meera Khan and Rattazzi, 9) and HGPRT (see Shin *et al.*, 14). The gel is blotted and incubated on a clean dry glass plate in a moist chamber at room temperature. For dehydrogenases the colored bands, and for IPO and PGK, the colorless bands, indicating the positions of enzyme protein, appear in the gel. After the bands are satisfactorily developed, the reaction is stopped by immersing the gel in 40% formaldehyde for 5 min. The gel is washed under tap water and preserved under distilled water at 4° for several months or made transparent for permanent record (17).

Staining: General principle. When the electrophoresed gel is treated with the reaction mixture containing an appropriate substrate and coenzyme together with an electron carrier (phenazine methosulfate) and tetrazolium salt (MTT), a zone of color due to diformazan production appears wherever a dehydrogenase occurs.

Specific staining. The specific stain recipe for each enzyme described in Table III is so arranged that the final volume of reaction mixture is just sufficient to treat one standard sheet (16 × 17 cm) of Cellogel.

RESULTS AND DISCUSSION

The electrophoretic patterns of G6PD[3], NADP–IDH, LDH, NAD–MDH, 6-PGD, HGPRT, IPO, PGM, and PGK on cellulose acetate gel are presented in Figs. 1–9 and summarized in Table IV. In view of the accumulated knowledge on such zymograms (see, for example, Shaw, 19, and Boone and Ruddle, 20), the figures are presumed to be self-explanatory. However, more remarkable features of individual electropherograms, if any, are described under legends to the respective figures.

The role of selectable as well as detectable biochemical genetic markers of the cultured mammalian cells in the genetic analysis of man via interspecific somatic cell hybrids, has been amply elucidated by several groups (3, 4, 13–16, 20, 21, 24–27).

In spite of the occurrence of remarkable evolutionary diversity, only 80–90% of the homologous enzymes studied were found to be electrophoretically distinguishable either between human and murine or between human and Chinese hamster phenotypes. This is certainly not surprising. MacCluer has calculated that about three quarters of all the single amino acid substitutions in a given polypeptide chain are electrophoretically undetectable (19). Moreover, the zymogram phenotype of a given protein is, after all, an expression of the interaction between the net charge on the functional molecule and its conformation and size—the changes that an evolving protein would eventually undergo.

Nevertheless, the "indistinguishable" wild interspecific enzyme phenotypes can be made distinguishable by employing a mutant cell, with an appropriate electrophoretic variant, as a fusion partner in the hybrid cell. To choose a suitable mutant cell line of a given partner is relatively easy for many enzymes, because of the fact that about 40% of the enzymes studied are found to be polymorphic both in man and mouse (28, 29) and it might be the same for other mammals including Chinese hamster. For example,

TABLE I

ELECTROPHORESIS BUFFER SYSTEMS

Buffer system	Composition		Recipe			pH
I	Tris	0.06140 M	Soln I[a]	Tris	72.8 g	7.5
(TEC "0.075")	EDTA	0.00400 M		Acid EDTA	9.4 g	
	Citric acid	0.01360 M		H_2O to	8000 ml	
			Soln II	Citric acid	31.5 g	
				Na_2EDTA	3.0 g	
				H_2O to	2000 ml	
II	Tris	0.03367 M	Soln I[a]	Tris	38.8 g	7.5
(TEC "0.04")	EDTA	0.00400 M		Acid EDTA	9.4 g	
	Citric acid	0.00633 M		H_2O to	8000 ml	
			Soln II	Citric acid	16.8 g	
				Na_2EDTA	3.0 g	
				H_2O to	2000 ml	
III	Tris	0.01833 M	Soln I[a]	Tris	19.4 g	7.5
(TEC "0.02")	EDTA	0.00400 M		Acid EDTA	9.4 g	
	Citric acid	0.00167 M		H_2O to	8000 ml	
			Soln II	Citric acid	4.2 g	
				Na_2EDTA	1.5 g	
				H_2O to	1000 ml	
IV	Na_2HPO_4	0.01 M	Soln I	$Na_2HPO_4 \cdot 2H_2O$	17.8 g	7.0
Citrate-PO_4 "0.01"	Citric acid	1.54×10^{-3} M		H_2O to	9900 ml	
			Soln II	Citric acid	5.0 g	
				H_2O to	100 ml	
The pH of solution I is adjusted by adding about 65 ml of solution II and the final volume of the buffer is brought to 10,000 ml with H_2O						
V	Na_2HPO_4	0.012 M	Soln I	$Na_2HPO_4 \cdot 2H_2O$	3.56 g	7.0
Phosphate "0.02"	NaH_2PO_4	0.008 M		H_2O to	1000 ml	
			Soln II	$NaH_2PO_4 \cdot H_2O$	2.76 g	
				H_2O to	1000 ml	
Solutions I and II are mixed in a 3:2 proportion to get the desired pH						
VI	Sodium veronal (0.04 M)		Sodium veronal		8.25 g	10.0
Veronal "0.04"				H_2O to	1000 ml	

[a] Solution I is adjusted to required pH with solution II.

the usual LDH B of man is electrophoretically indistinguishable from the LDH B of mouse. Ruddle *et al.* (21) have fused mouse cells with the lymphocytes from a human donor, heterozygous for an LDH B variant. The variant was readily distinguishable from the murine pattern and it made the human genetic analysis involving LDH B locus feasible. But the possibility of the existence of a linked cross-regulatory gene for LDH B could not be excluded in the man–mouse somatic cell hybrid system.

The problem could have been easily solved if a mutant homozygote were fused. But, to obtain a human homozygote for a rare mutation is extremely difficult if not impossible. Alternatively, a hybrid between a mammalian cell with distinguishable LDH B and the human fibroblast may be suitable. The LDH B of man and that of Chinese hamster are also indistinguishable whereas the LDH B of Syrian hamster is definitely slower than its human homologue on Cellogel electrophoresis (Fig. 10). Thus, the man–Syrian hamster somatic cell hybrid may be ideal for solving the LDH B

TABLE II

LIST OF REAGENTS FOR SPECIFIC REACTION MIXTURES

	Reagent	Concentration or recipe	pH	Storage temp	Usage
A.	Buffers				
A_1	Tris–HCl with EDTA	12.11 g Tris (1.0 M) and 0.149 g Na_2 EDTA (0.004 M) in 100 ml (pH adjusted with 1:1 HCl)	8.6		G6PD, 6PGD, PGK, LDH, IPO
A_2	Tris–HCl	4.36 g Tris (0.36 M) in 100 ml (pH adjusted with 1:1 HCl)	8.0		NAD–MDH, PGK, IDH
A_3	Tris–HCl with $MgSO_4$	0.666 g Tris (0.055 M) and 0.123 g $MgSO_4 \cdot 7H_2O$ (0.005 M) in 100 ml (pH adjusted with 1:1 HCl)	7.4		HGPRT
A_4	Tris–HCl	12.11 g Tris (0.1 M) in 1 liter (pH adjusted with 1:1 HCl)	9.5		HGPRT washing buffer
B.	Ionic solutions				
B_1	$CoCl_2$ (0.25 M) (BDH)	$CoCl_2 \cdot 6H_2O$ 5.95 g; H_2O to 100.0 ml			G6PD and 6PGD
B_2	KCN (0.003 M) (Fisher Sci. Co.)	KCN 19.5 mg; H_2O to 100.0 ml			PGM
B_3	$MgCl_2$ (0.1 M) (Merck)	$MgCl_2 \cdot 6H_2O$ 2.033 g; H_2O to 100.0 ml			PGM, PGK
B_4	$MnCl_2$ (0.0125 M) (BDH)	$MnCl_2 \cdot 4H_2O$ 0.247 g; H_2O to 100.0 ml			IDH, NAD–MDH
C.	Substrates				
C_1	PRPP (5-phosphoribosyl pyrophosphate) (Sigma)	2.0 mg in 7 ml of final mixture			HGPRT
C_2	Hypoxanthine-8-^{14}C (sp act 4.13 mCi/mmole). (New England Nuclear Corp., Boston, Mass.)	50 μCi/ml		−20°	HGPRT
C_3	6PG (gluconate-6-phosphate trisodium salt) (Boehringer)	10 mg/ml		−20°	6PGD
C_4	G-1-P (glucose-1-phosphoric acid dipotassium salt) BDH	16 mg/ml		−20°	PGM
C_5	G-1,6-diP(glucose-1,6-diphosphate tetracyclohexylammonium salt) (Boehringer)	1 mg/6ml		−20°	PGM
C_6	G6P(glucose-6-phosphate disodium salt) (Sigma)	20 mg/ml		−20°	G6PD
C_7	ICA(DL-isocitric acid triNa salt puriss) (Koch-Light)	85 mg/ml		−20°	ICD
C_8	3PG(3-phosphoglycerate tricyclohexylammonium salt) (Boehringer)	30 mg/ml		−20°	PGK
C_9	Sodium lactate solution containing 70–72% w/w $CH_3 \cdot CH(OH)COONa$ (BDH)	6.4 g/100 ml (0.4 M)			LDH
C_{10}	L-Malic acid (Baker)	2.681 g/10 ml (2 M) (pH adjusted with NaOH soln)	7.0	−20°	MDH

TABLE II—*Continued*

	Reagent	Concentration or recipe	pH	Storage temp	Usage
D.	Coenzymes				
D_1	NAD (β-nicotinamide adenine dinucleotide, free acid, reagent grade II) (Boehringer)	10 mg/ml		−20°	LDH, MDH
D_2	NADH (β-nicotinamide adenine dinucleotide, reduced form, disodium salt, reagent grade II) (Boehringer)	10 mg/ml		−20°	PGK
D_3	NADP (β-nicotinamide adenine dinucleotide phosphate, disodium salt) (Boehringer)	4 mg/ml		−20°	G6PD, 6PGD, PGM, IDH
E.	Miscellaneous				
E_1	ATP (adenosine-5′-triphosphate disodium salt) (Boehringer)	35 mg/ml		−20°	PGK
E_2	EDTA(ethylenediaminetetraacetic acid, free acid) (Baker)	15.78 g/liter (0.054 M) (neutralized with NaOH soln)	7.0		PGK and PGM
F.	Enzymes				
F_1	G6PD (glucose-6-phosphate dehydrogenase, reagent grade II, suspension) (Boehringer)	1 mg/ml		4°	PGM
F_2	GAPD (glyceraldehyde-3-phosphate dehydrogenase suspension) (Boehringer)	40 U/ml		4°	PGK
G.	Histochemicals				
G_1	MTT tetrazolium (Pearse) 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (Sigma)	2.0 mg/ml		4°	G6PD, IDH, LDH, MDH, 6PGD, IPO, PGM, PGK
G_2	PMS (phenazine methosulfate) (Sigma)	0.4 mg/ml		4°	As that of G_1

problem, provided such a hybrid is possible and also undergoes a preferential loss of human chromosomes from its genome, as in the case of man–mouse or man–Chinese hamster cell hybrids.

Since the realization that both the parental genomes are functional inside the somatic hybrid cell (30), the zymogram techniques have been extensively used to detect the (hybrid) nature of the fused cells and for genetic analysis of man *via* interspecific somatic cell hybridization. But certain criteria have to be satisfied in each case. In the *in vitro* mixtures of interspecific cell extracts, the IPO of man and mouse or man and Chinese hamster and the IDH of man and mouse spontaneously form the heteropolymeric molecules under the conditions which do not affect the artificial mixtures of other homologous enzymes studied in the same way. The intermediate bands seen in such instances (channels 3 and 4, Fig. 7 and channel 6, Fig. 2), therefore, cannot be helpful in the detection of the hybrid nature of the fused cell. An electrophoresis for PGM (Fig. 8) or PGK (Fig. 9) also will not be

TABLE III

LIST OF ENZYMES WITH PARTICULARS OF THEIR ELECTROPHORESIS AND STAINING ON CELLOGEL

Enzyme: trivial name (abbreviation; EC number; systematic name)	Buffer system	Run (hr)	Recipe for "staining" mixture[a]	Final volume (ml)	Incubation time (min)
Glucose-6-phosphate dehydrogenase (G6PD; EC 1.1.1.49; D-Glucose-6-phosphate: NADP oxidoreductase)	I (18)	2½	0.5 ml A_1 ; 0.2 ml D_3 , C_6 , G_1 , G_2 , B_1	1.5	10
Isocitrate dehydrogenase (NADP) (NADP-IDH; EC 1.1.1.42; *threo*-D_s-isocitrate: NADP oxidoreductase) (decarboxylating)	IV	3	1.0 ml A_2 ; 0.2 ml D_3 , C_7 , B_4 ; 0.4 ml G_1 , G_2	2.4	15
Lactate dehydrogenase (LDH; EC 1.1.1.27; L-Lactate: NAD oxidoreductase)	IV	2	1.0 ml A_1 , C_9 ; 0.4 ml D_1 , G_1 , G_2	3.2	5
Malate dehydrogenase (NAD-MDH; EC 1.1.1.37; L-Malate: NAD oxidoreductase)	II	2½	1.0 ml A_2 ; 0.2 ml C_{10} , B_4 , D_1; 0.4 ml G_1 , G_2	2.4	10
6-Phosphogluconate dehydrogenase (6-PGD; EC 1.1.1.44; 6-phospho-D-gluconate: NADP oxidoreductase) (decarboxylating)	I	3	1.0 ml A_1 ; 0.2 ml D_3 , C_3 , G_1 , G_2 , B_1	2.0	15
Hypoxanthine-guanine phosphoribosyl transferase (HGPRT; EC 2.4.2.8; IMP: pyrophosphate phosphoribosyl transferase)	V (13)	3	2.00 mg C_1 ; 6.65 ml A_3 ; 0.35 ml C_2	7.00[b]	60
Indophenol oxidase (IPO; see Brewer (1) for a detailed discussion on the genetics of this less-defined oxidase)	I	3	4.4. ml A_1 ; 0.8 ml G_1 , G_2	6.00[c]	60
Phosphoglucomutase (PGM; EC 2.7.5.1; α-D-glucose-1,6-diphosphate: α-D-glucose-1-phosphate phosphotransferase)	IV	2½	0.8 ml A_2 ; 0.2 ml B_3 , E_2 , B_2 , D_3 , C_5 ; 0.4 ml C_4 ; 5 μl F_1 ; 0.2 ml G_1 , G_2	2.60	15
3-Phosphoglycerate kinase (PGK; EC 2.7.2.3; ATP: 3-phospho-D-glycerate 1-phosphotransferase)	III	4	Stain I(fluorescent): 1.0 ml A_2 ; 0.2 ml B_3 , E_2 , D_2 , E_1 , C_8 ; 10 μl F_2	2.01	10[d]
			Stain II(histochemical): 2.00 ml A_1 , G_1 , G_2	6.00	5

[a] Reagents of these recipes are described in Table II and it is advisable to add them in the given order.

[b] DEAE-cellulose paper kept in contact with the gel is treated with the reaction mixture. After incubation in a moist chamber at room temperature for 60 min, the DEAE-cellulose paper is washed in the buffer A_4 and dried, and subjected to chromatography in A_4 itself. For details see Shin *et al.* (13).

[c] After treating uniformly and copiously with the reaction mixture the gel is incubated in an illuminated moist chamber till white bands appear against blue background indicating the position of the oxidase (1).

[d] In about 10 min of incubation of the gel in a moist chamber at room temperature, sharp quenched bands appear against the fluorescent background indicating the position of the enzyme when excited with a long wave uv light. The uv-excited gel can either be photographed directly before the bands get diffused (Fig. 9A) or the gel can be stained histochemically by which the PGK bands stay colorless while the background becomes dark blue (Fig. 9B). For this purpose, soon after the satisfactory appearance of quenched bands, the gel is copiously treated with Stain II, and incubated for about 15 min in an illuminated moist chamber.

useful for this purpose as neither forms heteropolymeric molecules (Table IV). Nevertheless, the segregation of a number of such human markers in a series of independent clones of a given fused cell line may indicate the hybrid nature of the cell in man–mouse or man–Chinese hamster cell system.

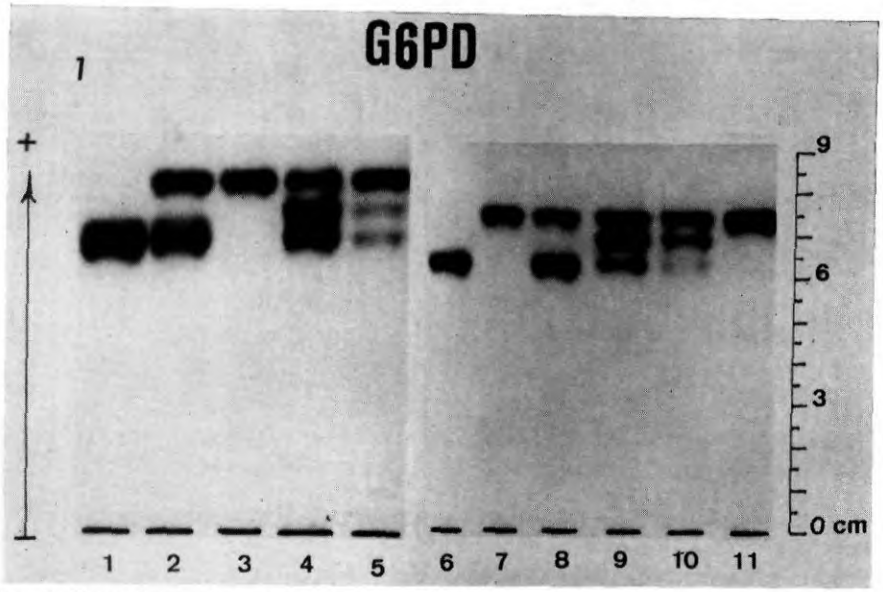

FIG. 1. Two electropherograms (1–5 and 6–11) stained for glucose-6-phosphate dehydrogenase showing human (1 and 6), mouse + human (2), mouse (3), man–mouse cell hybrid (4 and 5), Chinese hamster (7 and 11), Chinese hamster + human (8), and man–Chinese hamster cell hybrid (9 and 10) patterns. (Mixture of sonicates of different parental cell lines in Channels 2 and 8.) The gel 1–5 has longer run (2 hr 50 min) and incubation (30 min) than the gel 6–11 which has 2 hr 15-min run and 15-min incubation. Otherwise, G6PD's from Chinese hamster and mouse migrate identically in this system. Note the presence of intermediate bands indicating the positions of heterospecific heteropolymeric enzyme molecules (channels 4, 5, 9 and 10) of the somatic cell hybrids which are not formed in the mixtures of different parental cell lysates (channels 2 and 8). The differences in the relative intensities of the two homopolymeric and their heteropolymeric enzyme bands between samples 4 and 5 or 9 and 10 are striking. The significance and implications of these variations are discussed in detail by Miller *et al.* (13) and Meera Khan *et al.* (15), respectively.

By virtue of their formation of the heteropolymeric molecules (expressed in the form of intermediate bands in the zymograms), only in the hybrid cells but never in the mixtures of parental cell lysates under the conditions described, the G6PD (Fig. 1), or NAD-MDH (Fig. 4) electrophoresis in the man–mouse system and G6PD (Fig. 1), LDH A (Fig. 3), or 6-PGD (Fig. 5) electrophoresis in the man–Chinese hamster system are found to be useful in detecting the hybrid nature of these fused cells. Once the hybrid nature of the cells is established, all the enzyme markers either individually or collectively become useful in the genetic analysis of man.

Enzyme electrophoretic studies, on man–mouse somatic cell hybrids, have made it possible to study the gene–gene linkage relationships (21, 27) and gene–chromosome associations in man (24, 26) at the somatic cell level. The dispute regarding the origin of thymidine kinase (TK; EC 2.7.1.21) (25) or of HGPRT (14) in certain "aberrant" lines of man–mouse somatic cell hybrids, was settled by analyzing their electrophoretic patterns.

Acquisition of evidence for mitotic separation of genetic loci (G6PD and HGPRT), already known to be on the same chromosome (X) in man (13); or confirmation of a suggested association of a new marker (PGK) to the human X chromosome (15); or the utilization of such data in identifying the relative positions of loci for different enzyme markers (HGPRT, G6PD, and PGK) along the human X chromosome could be made possible only through the zymogram studies on man–mouse somatic cell hybrids (16). The same material and methods can

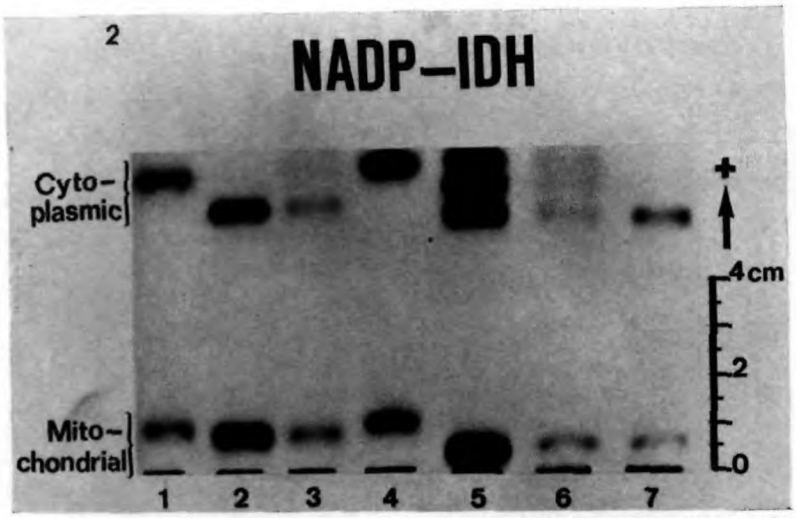

FIG. 2. Zymogram of NADP-dependant isocitrate dehydrogenase showing human (1 and 4), Chinese hamster (2), Chinese hamster + human (3), man–mouse cell hybrid (5), mouse + human (6), and mouse (7) patterns. In several series of man–Chinese hamster hybrid clones tested, no instance of the retention of human IDH was noticed by us (Westerveld *et al.*). In seven series of man–mouse hybrids only one (A_9/ADC of Miller *et al.*) has retained the gene for cytoplasmic component of human IDH (channel 5) in which an intermediate band is formed. A similar intermediate band is also formed in the mixture of lysates of cultured human and mouse fibroblasts (channel 6) but not in that of human and Chinese hamster (channel 3). The mitochondrial component of IDH from different sources appears to be distinguishable. Nevertheless, the human form of mitochondrial IDH is not seen either in the artificial mixtures (channels 3 and 6) or in the man–mouse hybrid (channel 5). (Mixture of sonicates of different parental cell lines in Channels 3 and 6.)

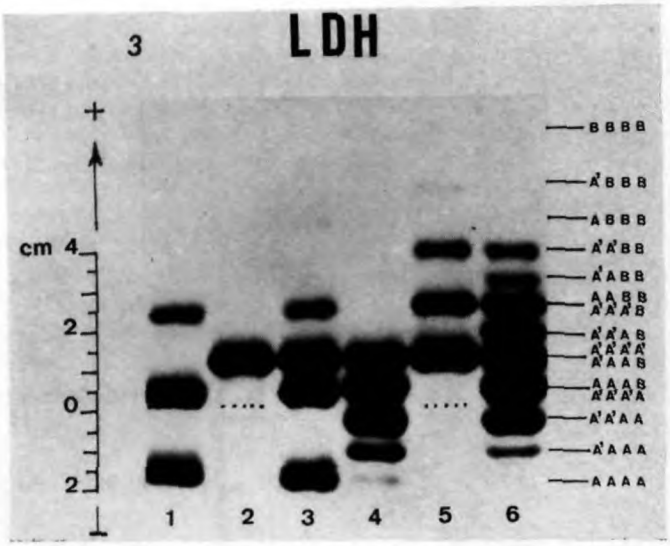

FIG. 3. Electrophoretic mobilities of lactate dehydrogenase isozymes on Cellogel, showing human (1), Chinese hamster DON (2), human + DON (3) and three different cloned man–Chinese hamster somatic hybrid (4, 5, and 6) cell patterns. (Mixture of sonicates of different parental cell lines in Channel 3.) The DON or any other Chinese hamster fibroblastic cell line has only one band corresponding to the Chinese hamster LDH A, i.e., A'A'A'A' (see also Fig. 10). The hybrid cells of channels 4, 5, and 6 appear to have retained the human LDH A, human LDH B, and human LDH A and B loci, respectively, in their genomes, in addition to the Chinese hamster locus for LDH A. The probable subunit structure of each band is indicated opposite to its position. A' in this figure represents the LDH A subunit of DON and A and B—the human LDH subunits. The significance of the occurrence of various patterns in different clones of man–Chinese hamster cell hybrids is discussed by Westerveld *et al.* (4).

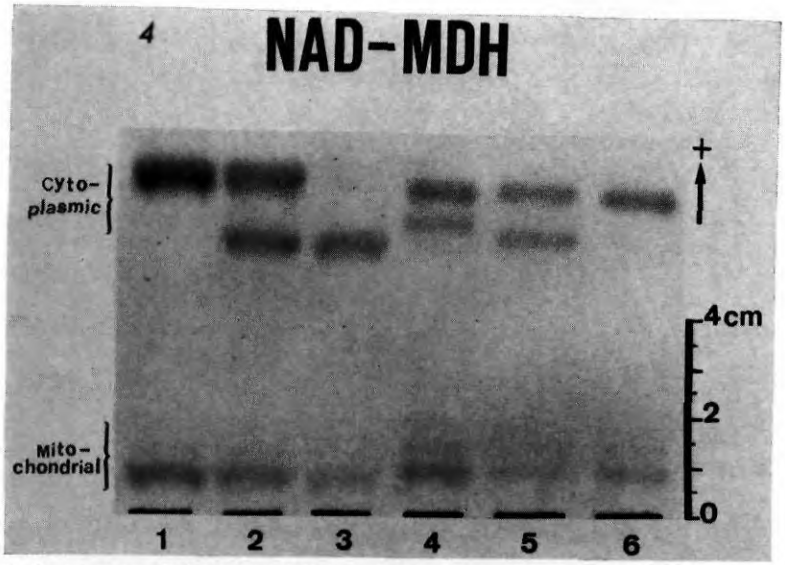

FIG. 4. Zymogram of the NAD-dependant MDH showing Chinese hamster (1), Chinese hamster + human (2), human (3), man–mouse cell hybrid (4), human + mouse (5), and mouse (6) patterns. (Mixture of sonicates of different parental cell lines in Channels 2 and 5.) As in the case of IDH, the human NAD–MDH is also lost in all the man–Chinese hamster hybrid cell lines tested. Nevertheless, an absence of linkage between the loci for cytoplasmic fractions of NADP–IDH and NAD–MDH in man is reported by Ruddle *et al.* (21), and our own observations on man–mouse somatic cell hybrids (Meera Khan and Siniscalco, unpublished data) are in agreement with this.

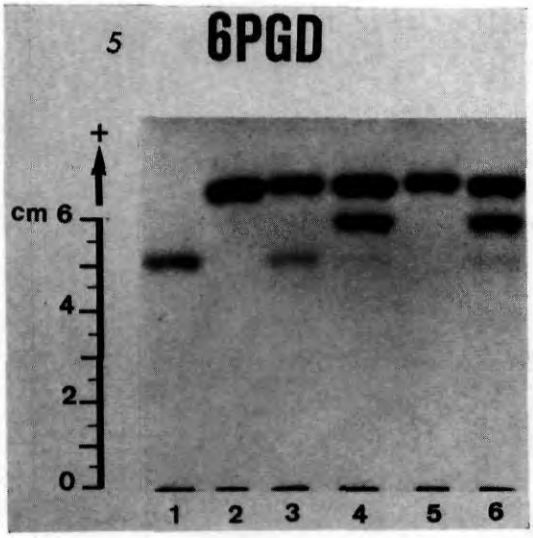

FIG. 5. Electrophoretic patterns of 6-phosphogluconate dehydrogenase in human (1), Chinese hamster (2 and 5), Chinese hamster + human (3), and man–Chinese hamster hybrid (4 and 6) cells cultured *in vitro*. (Mixture of sonicates of different parental cell lines in channel 3.) The human and murine (not shown in the figure) bands are not readily distinguishable. Therefore, the man–Chinese hamster somatic cell hybrid system became important in human genetic analysis involving the 6-PGD locus of man, while the man–mouse hybrid system turned out to be a less easy tool for this purpose.

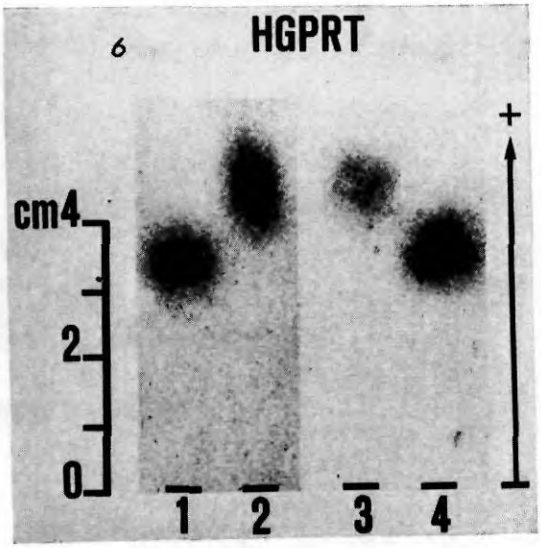

FIG. 6. Autoradiographs (1, 2 and 3, 4) showing the electrophoretic migration of HGPRT from Chinese hamster DON (1), human WBC (2), human fibroblast (3), and mouse L_{929} (4) cells. Chinese hamster enzyme migrates slightly slower than the mouse enzyme. The broader pattern of human WBC indicates contamination by RBC (see Shin *et al.*).

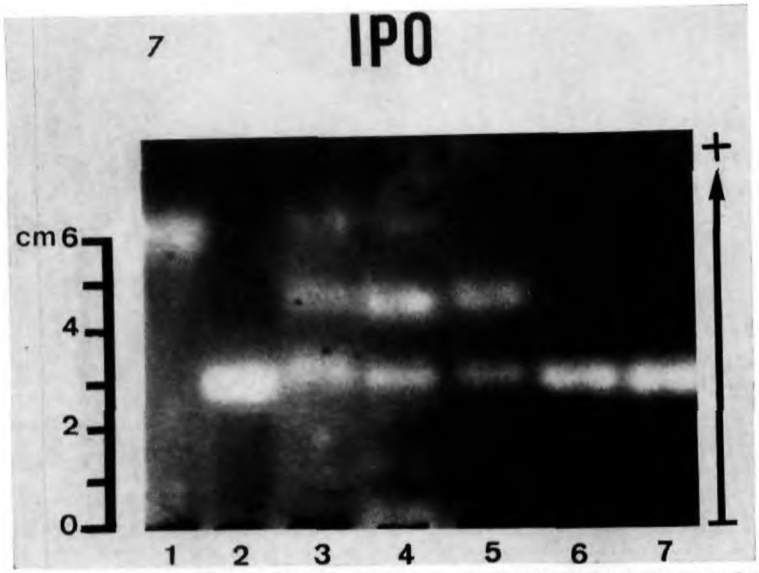

FIG. 7. Electrophoresis of indophenol oxidase on Cellogel. The gel presents human (1), mouse (2 and 7), human + mouse (3), human + Chinese hamster (4), man–Chinese hamster cell hybrid (5), and Chinese hamster (6) samples. Note the formation of an intermediate band in each of the mixtures (channels 3 and 4) of parental cell lysates. (Mixture of sonicates of different parental cell lines in Channels 3 and 4.)

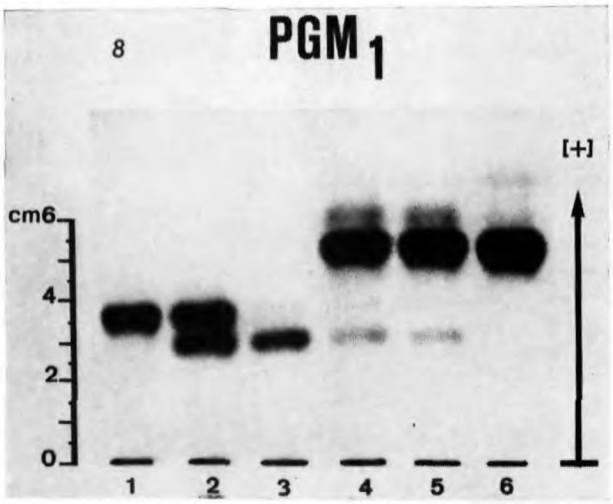

FIG. 8. Zymogram of phosphoglucomutase-1 showing mouse (1), mouse + human (2), human (3), man–Chinese hamster hybrid (4), human + Chinese hamster (5), and Chinese hamster (6) patterns. (Mixture of sonicates of different parental cell lines in Channels 2 and 5.)

be exploited fruitfully in detecting the new X-linked enzyme markers in man by screening the hybrid cells for all the possible enzymes whose genetics are not yet determined, because of the sparseness of their variation in the human populations so far explored (16). The same is true also to the TK gene carrying human chromosome E_{17-18}; because, this as well as the X are the only chromosomes in man proved at present to be carrying dependable selectable markers.

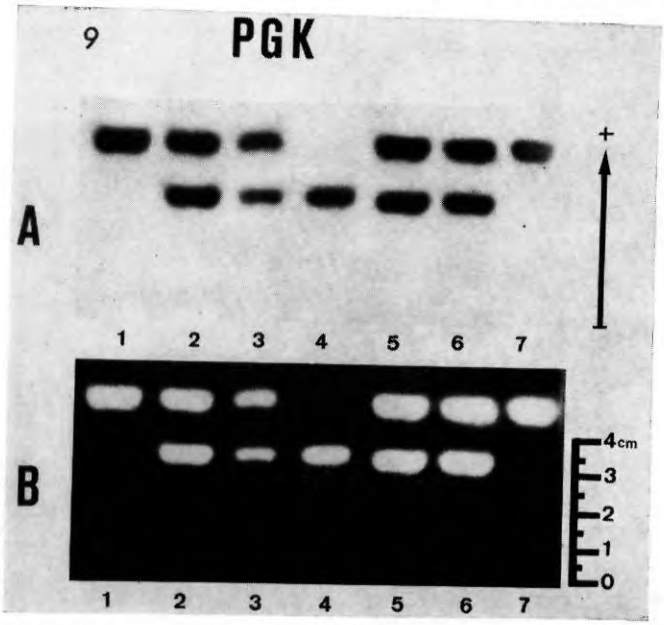

FIG. 9. A. Electropherogram of 3-phosphoglycerate kinase on Cellogel. Stain I: Quenched bands are seen against fluorescent background when excited by long-wave ultraviolet light, indicating the positions of PGK of mouse (1), human + mouse (2), man–mouse hybrid (3), human (4), man–Chinese hamster hybrid (5), human + Chinese hamster (6) and Chinese hamster (7). (Mixture of sonicates of different parental cell lines in Channels 2 and 6.) B. When Stain I is followed by Stain II the fluorescent background is stained dark blue while the nonfluorescent bands remain white. Note that the interspecific hybrids do not form detectable hetetopolymeric enzyme. An omission of 3-PG or ATP from the reaction mixture does not produce these nonfluorescent bands. The cultured fibroblasts derived from the skin of a boy deficient in red cell PGK (23) which showed only 2% of the normal fibroblastic activity as determined by the method of Blanchaer *et al.* (22), did not form a detectable nonfluorescent band even on prolonged incubation (15). ThePGK gene locus was suspected to be X-linked in man by Valentine *et al.* (23) during their study on an isolated kindred in which two boys, related through their female ancestors, were affected by red cell PGK deficiency and the fathers were normal while the mothers had intermediate expression for erythrocyte PGK activity. By utilizing a number of independant clones derived from man–mouse and man–Chinese hamster somatic cell hybrids via zymogram approach, it could be possible to show that the locus for *PGK* is on the X-chromosome in man (15).

The wild types of human and murine 6-PGD are found to be identical in their electrophoretic mobility on Cellogel in the buffer systems I, II, or III (Table I). But the 6-PGD patterns of man and Chinese hamster are readily distinguishable (Fig. 5). Recent studies, on man–Chinese hamster somatic cell hybrids indicated linkage between the loci for 6-PGD and PGM_1 in man (Westerveld and Meera Khan, in preparation). These two loci are thus, presumably located on the same human chromosome. But, on the other hand, family studies suggested nonlinkage between the loci for 6-PGD and PGM_1 in man (31, 32). This would imply that if at all the PGM_1 and 6PGD loci are present on the same chromosome in man, they are separated by more than 50 crossover units. Nevertheless, the possible occurrence of the so-called chromosomal coadaptability in the interspecific somatic cell hybrids may lead to the appearance of spurious linkages between markers on different chromosomes (26). As most of the human populations are polymorphic for both PGM_1 and 6-PGD, it should be easy

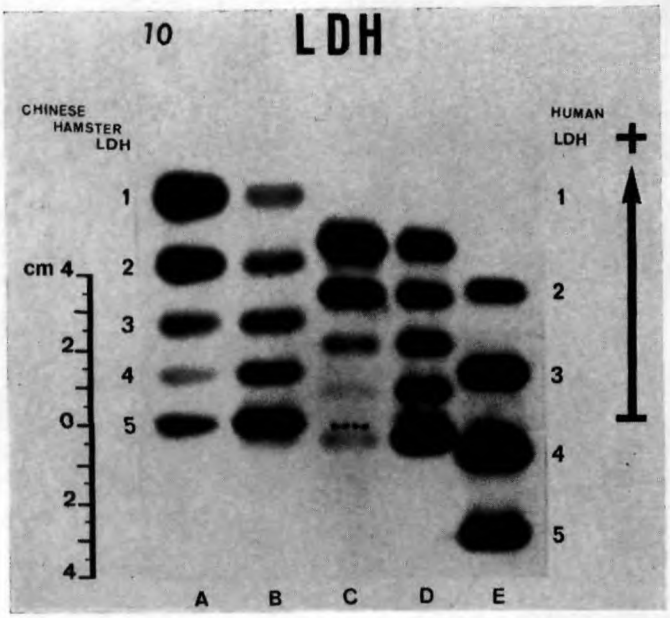

FIG. 10. LDH patterns on electrophoresis of the extracts from Chinese hamster heart tissue (A), Chinese hamster diaphragmatic tissue (B), Syrian hamster heart tissue (C), Syrian hamster diaphragmatic tissue (D), and cultured human fibroblasts (E).

TABLE IV

AN ANALYSIS OF THE ZYMOGRAM PATTERNS IN MAN–MOUSE AND MAN–CHINESE HAMSTER SOMATIC CELL HYBRIDS AND THEIR PARENTS

Enzyme	Man–mouse hybrid system				Man–Chinese hamster hybrid system			
	Human	Mouse	Human + mouse	Hybrid	Human	Chinese hamster	Human + Ch. hamster	Hybrid
G6PD	Slower	Faster	2 bands	3 bands	Slower	Faster	2 bands	3 bands
IDH (cytoplasmic)	Faster	Slower	3 bands[a]	3 bands	Faster	Slower	2 bands	[b]
LDH A	Slower	Faster	2 bands	[b]	Slower	Faster	2 bands	5 bands
LDH B	Indistinguishable		1 band	1 band[c]	Indistinguishable		1 band	1 band[c]
NAD-MDH (cytoplasmic)	Slower	Faster	2 bands	3 bands	Slower	Faster	2 bands	[b]
6-PGD	Indistinguishable		1 band	1 band[c]	Slower	Faster	2 bands	3 bands
HGPRT	Faster	Slower	2 bands	Not known	Faster	Slower	2 bands	Not known
IPO	Faster	Slower	3 bands[a]	[b]	Faster	Slower	3 bands[a]	3 bands
PGM_1	Slower	Faster	2 bands	[b]	Slower	Faster	2 bands	2 bands
PGK	Slower	Faster	2 bands	2 bands	Slower	Faster	2 bands	2 bands

[a] See Discussion.

[b] So far no example is found in which the loci of both the species were present together in our series of hybrid clones (Miller *et al.* (13); Westerveld *et al.* (4)).

[c] Presumed pattern.

to obtain donors with different phenotypic combinations. The fusion of lymphocytes from them with Chinese hamster cells should be of great use in confirming this linkage hypothesis and in studying the occurrence of somatic crossing over *in vitro*.

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REFERENCES

1. Brewer, G. J., *Amer. J. Hum. Genet.* **19**, 674 (1967).
2. Yoshida, A., *Proc. Nat. Acad. Sci. U. S. A.* **57**, 835 (1967).
3. Weiss, M. C., and Green, H., *Proc. Nat. Acad. Sci. U. S. A.* **58**, 1104 (1967).
4. Westerveld, A., Meera Khan, P. Visser, R. P. L. S., and Bootsma, D., *Nature London* (in press).
5. Ruddle, F. H., *in* "Control Mechanisms in Expression of Cellular phenotypes" (M. A. Padykula, ed.), Academic Press, New York (1970).
6. Hunter, R., and Markert, C., *Science* **125**, 1294 (1957).
7. Cavalli-Sforza, L. L., Zonta, L. A., Nuzzo, F., Bernini, L., de Jong, W. W. W., Meera Khan, P., Ray, A. K., Went, L. N., Siniscalco, M., Nijenhuis, L. E., van Loghem, E., and Modiano, G., *Amer. J. Hum. Genet.* **21**, 252 (1969).
8. Filippi, G., and Meera Khan, P., *Amer. J. Hum. Genet.* **20**, 564 (1968).
9. Meera Khan, P., and Rattazzi, M. C., *Biochem. Genet.* **2**, 231 (1968).
10. Lenzerini, L., Meera Khan, P., Filippi, G., Rattazzi, M. C., Ray, A. K., and Siniscalco, M., *Amer. J. Hum. Genet.* **21**, 142 (1969).
11. Rattazzi, M. C., Lenzerini, L., Meera Khan, P., and Luzzatto, L., *Amer. J. Hum. Genet.* **21**, 154 (1969).
12. Modiano, G., Bernini, L. F., Carter, N. D., Santachiara, A. S., Detter, J. C., Baur, E. W., Paolucci, A. M., Gigliani, F., Morpurgo, G., Santolamazza, C., Scozzari, R., Terrenato, L., Meera Khan, P., and Kanashiro, V., *Amer. J. Hum. Genet.* (in press).
13. Miller, O. J., Cook, P. R., Meera Khan, P., Shin, S., and Siniscalco, M., *Proc. Nat. Acad. Sci. U. S. A.* **68**, 116 (1971).
14. Shin, S., Meera Khan, P., and Cook, P. R., *Biochem. Genet.* **5**, 35 (1971).
15. Meera Khan, P., Westerveld, A., Grzeschik, K.-H., Deys, B. F., Garson, O. M., and Siniscalco, M., *Proc. Nat. Acad. Sci. U. S. A.* (in press).
16. Siniscalco, M., *in* "Control Mechanisms in Expression of Cellular Phenotypes" (M. A. Padykula, ed.), Academic Press, New York (1970).
17. Del Campo, G. B., *Clin. Chim. Acta* **22**, 475 (1968).
18. Rattazzi, M. C., Bernini, L. F., Fiorelli, G., and Mannucci, P. M., *Nature London* 213, 79 (1967).
19. Shaw, C. R., *Science* **149**, 936 (1965).
20. Boone, C. M., and Ruddle, F. H., *Biochem. Genet.* **3**, 119 (1969).
21. Ruddle, F. H., Chapman, V. M., Chen, T. R., and Klebe, R. J., *Nature London* **227**, 251 (1970).
22. Blanchaer, M. C., Williams, H. R., and Weiss, P., *Amer. J. Physiol.* **181**, 603 (1955).
23. Valentine, W., Hsieh, H., Paglia, D., Anderson, H., Baughan, M., Jaffé, E., and Garson, M., *N. Engl. J. Med.* **280**, 528 (1969).
24. Migeon, B. R., and Miller, C. S., *Science* **162**, 1005 (1968).
25. Migeon, B. R., Smith, S. W., and Leddy, C. L., *Biochem. Genet.* **3**, 583 (1969).
26. Nabholz, M., Miggiano, V., and Bodmer, W., *Nature London* **223**, 358 (1969).
27. Santachiara, A. S., Nabholz, M., Miggiano, V., Darlington, A. J., and Bodmer, W., *Nature London* **227**, 248 (1970).
28. Harris, H., *Proc. Roy. Soc. London Ser. B* **164**, 298 (1966).
29. Ruddle, F. H., Roderick, T. H., Shows, T. B., Weigl, P. G., Chipman, R. K., and Anderson, P. K., *J. Hered.* **60**, 321 (1969).
30. Weiss, M. C., and Ephrussi, B., *Genetics* **54**, 1111 (1966).
31. Edwards, J. H., and Wingham, J., *Ann. Hum. Genet.* **30**, 233 (1967).
32. Weitkamp, L. R., Guttormsen, S. A., Shreffler, D. C., Sing, C. F., and Napier, J. A., *Amer. J. Hum. Genet.* **22**, 216 (1970).