long been a favorite species for planting but this practice will reduce water supplies. On watershed 1, water available for downstream use in 1972 was reduced by 23.7×10^6 liters by converting just 16 hectares from deciduous hardwood to white pine. Identical water yield reductions would not be expected everywhere because of differences in climate and vegetation. But a summary of interception by conifers in North America (*9*) indicates greater interception loss for pine species and other conifers than for deciduous forests. Thus, since the evaporative processes involved are universal, a trend toward streamflow reductions when deciduous hardwood stands are converted to pine might be expected in other regions.

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11 February 1974; revised 9 April 1974

Human Serum Albumin Phenotype Activation in Mouse Hepatoma–Human Leukocyte Cell Hybrids

Abstract. *Murine hepatoma cells that secreted mouse serum albumin were fused with human leukocytes that did not produce albumin. The resulting hybrids secreted both mouse and human serum albumin, as demonstrated by immunoelectrophoretic techniques. The activation of the human genome suggests that mapping genes governing specialized functions in somatic cell hybrids may be accomplished by using adifferentiated human cells as a parental line.*

Interaction between cells in different epigenetic states has been examined in a variety of somatic cell hybrid combinations (*1, 2*). Three general results have been reported: (i) The specialized phenotype continues to be expressed (*3*), (ii) it is extinguished (*4*), or (iii) the specialized phenotype is activated in the allogeneous genome (*5*). These kinds of experiments do not directly provide information about the process of cellular differentiation, but they do provide opportunities for the analysis of phenotype modulation in specialized cells. We report here the activation of the human serum albumin (HSA) phenotype in hybrid cells between a murine hepatoma line and human leukocytes. Furthermore, we propose that such allogeneous hybrid combinations between a mouse differentiated cell line and diploid human fibroblasts or leukocytes can be used to map human genes governing specialized phenotypes. Possibly such hybrids may also provide insight regarding molecular mechanisms that regulate gene expression.

Murine hepatoma cells were adapted to in vitro growth by the procedure described by Buonassisi *et al.* (*6*). The tumor from which the cells were derived is the BW 7756 hepatoma carried in C57 leaden mice (Jackson Laboratory, Bar Harbor, Maine). Hepa 1a, which is a clonal population deficient in hypoxanthine guanine phosphoribosyltransferase (HGPRT), was used as the murine parental line. This enzyme-deficient line was obtained by treating 10^6 hepatoma cells with 10 μg per milliliter of thioguanine in medium MAB 87/3 until colonies were sufficiently large to be isolated. One such colony, Hepa 1a, was expanded and then shifted to medium containing 30 μg per milliliter of thioguanine. Activity for HGPRT in these cells was measured in two ways. (i) Hepa 1a failed to grow in medium containing aminopterin, hypoxanthine, and thymidine [see (*7*)]. (ii) No enzymatic activity could be seen when cell extracts were subjected to electrophoresis and then examined by autoradiography, according to the method of Tischfield *et al.* (*8*). Hepa 1a cells have retained the ability to express some liver-specific traits in vitro (*9, 10*); of most interest is the synthesis and secretion of mouse serum albumin (MSA). The MSA secretion rate is approximately 230 ng of albumin per milligram of cellular protein per hour (*11*). Serum albumin secretion appears to be restricted solely to hepatocytes (*12, 13*). Cell line Hepa 1a has been examined for the presence of other hepatic traits, and Es-2, a liver-specific esterase (*14*), was shown to be present. Activity for alcohol dehydrogenase, xanthine oxidase, and aldolase B could not be demonstrated by starch-gel electrophoresis. The karyotype of Hepa 1a differed from the normal diploid cell; four to six bi-armed chromosomes were present and the modal chromosome number was 58. In addition, a long acrocentric chromosome containing interdigitated heterochromatic regions served as a useful marker.

The human parental cells were peripheral leukocytes from a normal male. Before hybridization, leukocytes were separated from the whole blood according to the method of Bodmer *et al.* (*15*), but with the substitution of Plasmagel (Roger Bellon Laboratories, Neuilly, France) for the dextran. After fusion (*16*), colonies grew within 8 weeks, and five of these were isolated and designated Hal 3, Hal 5, Hal 6, Hal 7a, and Hal 7b. The latter two, 7a and 7b, appeared in the same culture flask and most probably arose from a single fusion event, since they have similar chromosome and phenotype constitutions.

To verify the hybrid nature of the Hal populations, cell extracts were analyzed by enzyme electrophoresis. All hybrids expressed HGPRT activity characteristic of the human parent (see Fig. 1A for the electrophoretic mobility of this enzyme) (*17*). Cell extracts were tested for 25 additional enzymes whose electrophoretic mobilities differ between mouse and man (see legend, Table 1). The mouse forms of all enzymes were invariably present, but only a few human phenotypes were retained in any one hybrid (Table 1). No more than two human autosomally inherited markers plus the X-linked genes were seen in any Hal line, suggesting that most of the human chro-

Table 1. Human enzyme phenotypes and chromosomes retained by Hal hybrids. The enzymes examined electrophoretically (*26*) in hybrid cell extracts were adenylate kinase, adenosine deaminase, alcohol dehydrogenase, esterase-2, glutamic oxaloacetic transaminase, glucose-6-phosphate dehydrogenase (GPD), glucose phosphate isomerase (GPI), hypoxanthine guanine phosphoribosyltransferase (HGPRT), mitochondrial superoxide dismutase (SOD-1), cytosol superoxide dismutase (SOD-2), lactate dehydrogenase A, lactate dehydrogenase B, malic enzyme (ME-1), malate dehydrogenase, mannose phosphate isomerase, nucleoside phosphorylase (NP), peptidases A, B, C, and D, phosphoglucomutase-1, phosphoglycerate kinase (PGK), and xanthine oxidase.

Cell line	Human isozymes present	Modal chromosome number	Human chromosomes expected from isozyme data*	Human chromosomes present from cytological examination†
Hepa 1a		58		
Hal 3	GPI HGPRT, GPD, PGK	59	19, X	X (13/36)
Hal 5	HGPRT, GPD, PGK		X	Not done
Hal 6	SOD-1, ME-1, SOD-2 HGPRT, GPD, PGK	60	6, X, 21	6 (17/47), X (29/47), 8 (23/47)
Hal 7a	NP, ME-1 HGPRT	106	Rearranged 6 Rearranged X 14	None (0/39)
Hal 7b	NP HGPRT, GPD, PGK	110	14, X	X(2/29)

* The gene for glucose phosphate isomerase activity has been assigned to chromosome 19 by McMorris *et al.* (*27*). Chromosome 6 carries the loci governing the expression of SOD-1 and ME-1 (*18, 19*). Tan *et al.* (*28*) have located the gene for SOD-2 on chromosome 21. The gene for nucleoside phosphorylase activity has been located on chromosome 14 (*24*). † The numerator of the ratio in parentheses represents the number of cells in which the chromosome was observed. The denominator is the total number of cells examined.

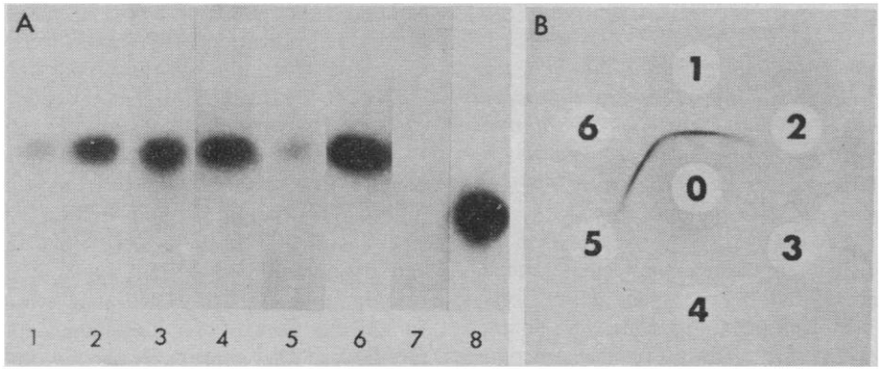

Fig. 1. (A) Composite photograph showing the electrophoretic separation of human and mouse hypoxanthine guanine phosphoribosyltransferase (HGPRT). The origins not shown in the figure are the same for all samples. Cell extracts were prepared and separated by starch-gel electrophoresis (*26*), and the enzyme activity was visualized by autoradiography (*8*). All hybrids show the retention of the fast migrating human form of HGPRT. (Top) Anode; (bottom) cathode. 1, Hal 3; 2, Hal 5; 3, Hal 6; 4, Hal 7a; 5, Hal 7b; 6, human tissue culture cells KB; 7, Hepa 1a ($HGPRT^-$); and 8, Hepa 1, the HGPRT-positive cell line from which Hepa 1a was derived. (B) Identification of human serum albumin (HSA) by double immunodiffusion. Confluent cultures (3×10^6 to 5×10^6 cells per Falcon flask, 75 cm^2) were incubated for 24 hours in serum-free medium. The growth medium was harvested, dialyzed, concentrated approximately 200-fold, and analyzed by double immunodiffusion (*11*). Identity of the protein secreted by the hybrid lines Hal 7a and purified HSA is demonstrated by the complete fusion of the precipitin bands formed between antiserum to HSA (0) and supernatant medium from Hal 7a (1) and HSA (6). Cell-free medium (4) and growth medium from the parental line Hepa 1a (5), both concentrated 200 times are shown as controls to demonstrate the absence of cross-reacting materials. The absence of cross-reaction between antiserum to HSA and murine serum albumin (MSA) was tested for MSA concentrations ranging from 10 to 10^3 micrograms per milliliter by immunoprecipitation. 0, Monospecific crystalline goat antiserum to HSA (Schwarz/Mann, New York), 10 μg; 1, Hal 7a supernatant; 2, Hepa 1a supernatant; 3, MSA, ethanol extraction of Cohn fraction V, 5 μg; 4, cell-free growth medium; 5, fetal calf serum (Flow Laboratories, Rockville, Md.), 10 μg protein; 6, HSA, Cohn fraction V, 5 μg purified by trichloroacetic acid precipitation and solubilization in acidic ethanol according to the procedure of Korner and Debro (*29*).

mosomes were lost from the hybrids. Furthermore, Hal 7a exhibited a disruption of known linkage relationships in that the X-linked genes for glucose-6-phosphate dehydrogenase (GPD) and phosphoglycerate kinase (PGK) were absent although the human form of HGPRT was present. A second indication of chromosomal rearrangement in Hal 7a was the discordant segregation of malic enzyme (ME-1) from mitochondrial superoxide dismutase (SOD-2). Both loci have been assigned to chromosome 6 (*18, 19*).

The chromosomal composition of Hal 3, 6, 7a, and 7b was examined by using quinacrine mustard–stained preparations that were subsequently stained for centric heterochromatin by the formamide-Giemsa method of Dev *et al.* (*20*). The modal numbers (Table 1) of Hal 3 ($N = 59$) and Hal 6 ($N = 60$) were similar to that of Hepa 1a ($N = 58$). Hal 7a and 7b, however, appeared to have a double input of the hepatoma genome. In these hybrid lines, two Hepa 1a acrocentric marker chromosomes per cell were observed in more than 90 percent of the cells examined.

Table 1 enumerates the human chromosomes observed in each hybrid analyzed and compares the observed data with the expected chromosomal composition based on isozyme information. Hal 3 retained the human X chromosome in 30 percent of the 38 cells examined. Although glucose phosphate isomerase (GPI) is present in Hal 3, chromosome 19 was not observed. Hal 6 retained chromosomes 6 and X in addition to 8, which has yet to be associated with a linkage group; chromosome 21 was not observed in Hal 6. In colony 7a, no structurally intact human chromosome could be identified, nor was any portion of a human chromosome observed in the karyotype. In 7b only 2 cells in 28 contained a normal human X chromosome.

The hybrid lines all lacked activity for xanthine oxidase, alcohol dehydrogenase, and aldolase B. Esterase 2 was present and MSA was secreted by all hybrid clones. Therefore, both murine hepatic characteristics present in Hepa 1a (MSA and Es-2) continued to be expressed in the hybrids. In addition to the synthesis of MSA, the hybrid clones Hal 7a and 7b also secreted HSA (Fig. 1B). The production of HSA is the first demonstration of the activation of a human hepatic phenotype in somatic cell hybrids and ex-

tends the work of Peterson and Weiss (*5*) who demonstrated activation of the MSA phenotype in a rat hepatoma × mouse fibroblast hybrid system. Quantitative measurements of MSA and HSA were made by using the electroimmunodiffusion procedure of Laurell (*21*) and employing monospecific non-cross-reacting rabbit antiserum to MSA and goat antiserum to HSA (*11*). The absence of cross-reaction between antiserum to HSA and MSA allows the determination of small amounts of HSA in the presence of excess MSA. The amount of HSA secreted by the hybrid lines Hal 7a and 7b was approximately 1 percent of the total serum albumin produced by these cells as determined by the Laurell procedure [see (*11*)]. At least two possible explanations exist for this observation: (i) the Hal 7a and 7b populations are mosaic with regard to HSA production and (ii) the intercellular milieu is suboptimal for the expression of HSA.

The activation of the human albumin gene plus the segregation of the human chromosomes provides a genetic system for the assignment of the albumin locus to a particular chromosome. Because the number of hybrid populations was small, we have been prevented from making such an assignment, but our early results provide some clues. Human enzyme phenotypes for adenylate kinase, adenosine deaminase, mitochondrial glutamic oxaloacetic transaminase, GPI, lactate dehydrogenase A, lactate dehydrogenase B, malate dehydrogenase, mannose phosphate isomerase, phosphoglucomutase-1, and peptidases A, B, C, and D were absent in the HSA-positive clones. Alternatively, phenotypes GPD, HGPRT, mitochondrial superoxide dismutase (SOD-1), SOD-2, ME-1, and PGK were expressed in HSA-negative clones. Therefore, genes governing these phenotypes are most probably not syntenic with the albumin locus. It is known that the albumin locus is autosomal from its pattern of inheritance in man (*22*) and mouse (*23*). One autosomal marker, nucleoside phosphorylase (NP), correlates with HSA in Hal 7a and 7b. In subsequent generations of Hal 7a both NP and HSA were lost while ME-1 was retained. It is well established from somatic genetic studies that NP can be assigned to chromosome 14 (*24*). Thus, the data are consistent with, but in no way prove, an association between HSA and chromosome 14. This possibility should be further tested in family and cell genetic investigations. Localization of the albumin gene is necessary for identifying the genetic relationships between the structural locus and any presumptive control loci.

The results reported here provide the first data on human phenotype activation in a man-rodent combination that segregates human chromosomes. In one other instance of phenotype activation, the parental cells were both rodent in origin and tended to conserve both chromosome sets (*5*). These rodent hybrid combinations have suggested the importance of genome dosage in phenotype activation, since in all instances activation was observed in hybrids that possessed two genomes from the differentiated parent to one from the adifferentiated parent. The only populations in our experiments that showed activation possessed two hepatoma genomes, which is consistent with the results previously reported (*5*). Expression of HSA is consistent with the hypothesis that the murine genome contributes activators to those hybrids retaining the albumin structural gene. An alternate hypothesis (*4*) would postulate the segregation of human repressors from the hybrids, thereby allowing the expression of the human albumin locus.

The amount of MSA secreted in the Hal 7a and 7b hybrids was measured by electroimmunodiffusion (*21*). Both hybrids were found to secrete MSA on the order of 100 to 250 ng mg^{-1} $hour^{-1}$, which is not significantly different from the rate of 150 to 230 ng mg^{-1} $hour^{-1}$ observed in Hepa 1a (*11*). The continued expression of MSA may reflect the absence of intergenomic repressors or the segregation of these elements from the hybrids. Specific repressor genes of this type have not yet been demonstrated, but mouse-human hybrids that segregate the adifferentiated genome provide an approach to this problem.

A final attribute of the Hal 7a and 7b cells deserves brief mention. The human chromosomes in these clones were extensively rearranged, which introduces the possibility that the human albumin locus was separated from normally adjacent chromosomal regions and integrated into the mouse genome. Such mouse-human translocations have been previously described (*25*).

In this report, we have demonstrated the activation of the human albumin gene derived from a cell type in which it is normally not expressed. We believe that this system may offer opportunities for genotype testing in clinical medicine. Biopsy of certain tissue types such as liver, brain, and so forth, presents risks to the adult patient; prenatal samples of such tissues are completely unavailable. The demonstration of gene activation in leukocytes suggests that a large repertory of gene products in man is potentially available for examination by hybrid activation. The realization of these possibilities will depend on the development of appropriate differentiated cell lines, phenotype assays, and hybrid selection systems. Such developments are essentially methodological and should be easily achieved in the context of current knowledge.

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30. This work was supported by grants from PHS (GM 09966) and NSF (GB 34303). H.P.B. is the recipient of awards from the Swiss National Foundation and the PHS (TW-01896). We thank Elizabeth Nichols, Annelis Bernhard, and Patricia Eager for their excellent technical assistance.

11 March 1974; revised 14 May 1974

Amino Acid Difference Formula to Help Explain Protein Evolution

Abstract. *A formula for difference between amino acids combines properties that correlate best with protein residue substitution frequencies: composition, polarity, and molecular volume. Substitution frequencies agree much better with overall chemical difference between exchanging residues than with minimum base changes between their codons. Correlation coefficients show that fixation of mutations between dissimilar amino acids is generally rare.*

I present here an improved formula for difference between amino acids which identifies the chemical factors that individually correlate best with evolutionary exchangeability of protein residues. I also estimate the extent to which observed exchanges can be explained by conservation of these factors.

Disagreement exists over what mainly directs gene evolution at the molecular level. Essentially the controversy is between randomness and physicochemical determinism. Randomness proponents (*1*) believe that proteins have evolved by chance fixation of "neutral mutations" (substitutions of one amino acid for another such that the original and substituted genes have equal adaptive values). The other viewpoint is that physicochemical forces are the principal determinants of molecular evolution. There has not been enough effort, though, to specify these forces quantitatively. I now relate overall chemical difference, approximated from side chain properties, to evolutionary difference between amino acids, implied by their "mutation rates." Although precise measures are yet to come, Dayhoff (*2*) and McLachlan (*3*) have estimated frequencies of exchange between protein residues. For the correlations in this report we use relative substitution frequency (*RSF*) of McLachlan, which is the largest sampling so far (*3*).

Methods for assessing total difference between amino acids are few. Sneath's index (*4*) contains too many characters for satisfying correlations [although Clarke (*5, 6*) improved this among amino acids whose codons have two common bases by judicious weighting of the characters], while Epstein's formulas (*7*) considering only size and polarity class yield identical differences for many pairs of amino acids. The number of base changes (in the messenger RNA's, derived by decoding the proteins) needed to give the same amino acid sequence is used to construct phylogenetic trees. But amino acid substitution frequencies cannot be rationalized this way (*2, 3*). Further, the minimum base change method poorly reflects homology and consistently underestimates the total number of fixations inferred from phyletic data (*8*). The Sneath or Epstein difference correlates better against *RSF* than does the minimum number of base changes, but their correlation coefficients remain weaker than −.5 (see below). This leaves considerable room for chance determination of residue exchanges.

Several amino acid side chain properties correlate appreciably with *RSF*. The three strongest correlators are composition, polarity, and molecular volume. These last two properties are from published data [see (*9*)]. Composition, *c*, is defined as the atomic weight ratio of hetero (noncarbon) elements in end groups or rings to carbons in the side chain. Such ratios are a simple and sensitive way of reflecting composition differences between amino acids. As an example, for the

Table 1. Values for properties in amino acid difference formula and correlation results (*10*). The correlation coefficient R_{19} was obtained from linear regression of log *RSF* on *D* within each group of 19 amino acid pairs; R_{190} was obtained with all 190 pairs by regression of log *RSF* on *D* given by indicated single property or combination of properties; R_S is the Spearman rank correlation coefficient (*12*). For interproperty correlations, the 190 differences for one property were ranked against those for the other property; the interproperty R_S values are *cp*, .435; *cv*, .092; and *pv*, .008. The $\overline{D}$ below each property column is the average chemical distance given by that property alone; thus $\overline{D}_c = \Sigma[(c_i - c_j)^2]^{1/2}/190$. The inverse mean weighting factors are $\alpha = (1/\overline{D}_c)^2 = 1.833$; $\beta = (1/\overline{D}_p)^2 = 0.1018$; $\gamma = (1/\overline{D}_v)^2 = 0.000399$.

Amino acid	Property			$-R_{19}$	Formula	$-R_{190}$	$-R_S$
	c	*p*	*v*				
Ser	1.42	9.2	32	.76	*c*	.49	.49
Arg	0.65	10.5	124	.68	*p*	.47	.55
Leu	0	4.9	111	.92	*v*	.37	.33
Pro	0.39	8.0	32.5	.67			
Thr	0.71	8.6	61	.63	*cp*	.61	
Ala	0	8.1	31	.75	*cv*	.61	
Val	0	5.9	84	.86	*pv*	.63	
Gly	0.74	9.0	3	.66			
Ile	0	5.2	111	.89	*cpv*	.72	.765
Phe	0	5.2	132	.83			
Tyr	0.20	6.2	136	.64			
Cys	2.75	5.5	55	.31			
His	0.58	10.4	96	.53			
Gln	0.89	10.5	85	.79			
Asn	1.33	11.6	56	.87			
Lys	0.33	11.3	119	.76			
Asp	1.38	13.0	54	.93			
Glu	0.92	12.3	83	.82			
Met	0	5.7	105	.58			
Trp	0.13	5.4	170	.58			
$\overline{D}$	0.739	3.134	50.06				