Proc. Natl. Acad. Sci. USA
Vol. 75, No. 2, pp. 936–940, February 1978
Genetics

Expression of human adenosine deaminase after fusion of adenosine deaminase-deficient cells with mouse fibroblasts

(genetic regulation/somatic cell hybridization/isozymes)

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Communicated by John W. Littlefield, November 11, 1977

ABSTRACT Two human choriocarcinoma cell lines were shown to be deficient in adenosine deaminase (ADA; adenosine aminohydrolase, EC 3.5.4.4) such that they did not produce bands on starch gels after electrophoresis and histochemical staining. Radiometric assay indicated that their ADA specific activity was approximately 2% that of HeLa (human) cell controls. Subclone analysis of one of the lines indicated that this deficiency was representative of individual cells of the line. After fusion of these cells with mouse fibroblasts having high ADA activity, most independently isolated hybrid clones expressed one of two, or both, additional (to the mouse) bands of ADA activity after electrophoresis. The expression of these extra bands in hybrids was dependent upon actual fusion. The phenomenon was observed in 30 of 45 independently derived hybrid clones from four different fusion experiments involving two different parental lines from each species. The pattern of appearance of the extra bands in independent hybrid clones and the tendency of a hybrid clone to lose one of the extra bands through subsequent passages suggests that the bands were the products of human genetic material. The extra bands electrophoretically comigrated with human ADA 1 and 2 from human *ADA-1-2* heterozygotes and the faster-migrating of the two extra bands comigrated with human ADA 1 from HeLa cells. Therefore, we suggest that the bands appearing in hybrids are the products of the *1* and *2* alleles of the human *ADA* locus. The human cells used for fusion were deficient in ADA activity but contained the genetic information for ADA 1 and 2. Fusion with mouse cells having ADA activity resulted in the activation of both human gene products coded for on separate homologous chromosomes. We conclude that the human *ADA* locus is under manipulatable genetic regulation.

The enzyme adenosine deaminase (ADA; adenosine aminohydrolase EC 3.5.4.4) has attracted recent attention with the discoveries of abnormal levels in patients with genetic diseases. Low levels are associated with severe combined immunodeficiency disease (1–7), and elevated ADA with hereditary hemolytic anemia (8). Approximately 10% of the human population is heterozygous for ADA as detectable by starch gel electrophoresis—that is, two allozymes are detected, the product of allele *ADA-1* migrating farther during electrophoresis than the product of *ADA-2* (9). Because the enzyme is composed of a single subunit (is monomeric), there are no bands of intermediate migration. Except for some rare types, the remainder of us are *ADA-1-1* homozygotes, producing only the faster band. Here we report the results of somatic cell hybridization experiments involving a human choriocarcinoma cell line with essentially no ADA activity. Extracts of these cells are like those from cells of individuals with severe combined immunodeficiency disease (1) in that ADA is undetectable on starch gels. The choriocarcinoma cells were fused to mouse cells having substantial activity. In hybrid clones the products of both human *ADA-1* and *ADA-2* alleles were easily visible on gels.

The data presented support the hypothesis that ADA activity is under genetic regulation. The human cells used are deficient in ADA activity but have the genetic information for ADA 1 and 2. Fusion with mouse cells having activity results in the activation of both human gene products coded for on separate homologous chromosomes.

MATERIALS AND METHODS

Cell Lines. Both human cell lines used (JEG-3 and HCC) were derived from a choriocarcinoma of a single individual. The choriocarcinoma cells were initially carried through animals and then put in culture. JEG-3 is a cloned line established from those cells (10). HCC is a segregant population of JEG-3 that was carried separately in animals and placed into culture, but not cloned. Two mouse fibroblast lines were used: $LMTK^-$ clone 1D (Cl 1D), a line deficient in thymidine kinase (EC 2.7.1.21) (11) and A9, a line deficient in hypoxanthine phosphoribosyltransferase (EC 2.4.2.8) (12). HeLa strain S_3 (human) was supplied by Judith Clarkson of the M. D. Anderson Hospital and Tumor Institute.

Cell Culture and Subcloning. The JEG-3, HCC, HeLa, and Cl 1D cell lines were grown in F-12 medium with modifications to augment glucose and certain salts (F-12 Mod—available from GIBCO as F-12 modified by Bordelon). The F-12 Mod is supplemented with 13% horse serum, 2.5% fetal calf serum, penicillin at 100 units/ml, and streptomycin at 100 μg/ml (all from GIBCO). The A9 cells were grown in high-glucose Dulbecco's medium (GIBCO) supplemented with 13% fetal calf serum and antibiotics as listed above. Cells were kept in a 37° humidified incubator gassed with 5% CO_2 and 95% air. JEG-3 and HCC were subcultured with a 1 to 2 split to retain homogeneity and correct passage numbers. JEG-3 cells were subcloned using microtest plates (Falcon). The cells were trypsinized and resuspended in medium with serum at a concentration of approximately 10 cells per ml, and 0.1 ml was added to each well of a microtest plate. The plates were incubated for 2 hr to allow attachment and viewed with an inverted phase microscope, and wells with single cells were marked. Cells in such a well were grown up and transferred to 30-mm petri dishes for establishment of subclones.

Cell Fusion. Four different fusion experiments were conducted: JEG-3 × Cl 1D, HCC × Cl 1D, JEG-3 × A9, and HCC × A9. Cells were fused in suspension, using β-propiolactone-inactivated Sendai virus. Cells were trypsinized to a single cell suspension, and 10^6 cells were mixed for each fusion. The cells were chilled with 2000 hemagglutinating units (HAU) of virus,

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Abbreviation: ADA, adenosine deaminase.

incubated 2 h at 37°, and dispensed into 20 100-mm petri dishes containing F-12 Mod. Two days later the medium was exchanged for F-12 Mod + hypoxanthine, aminopterin, and thymidine (HAT) to select against the mouse cells (13). Clones were selected on the basis of morphology, removed with cloning rings, and grown on HAT medium. Details of the fusion have previously been reported (14).

Electrophoretic Analysis. Starch gel electrophoresis was conducted on the homogenates of clones surviving hybrid selection conditions and was followed by histochemical staining to detect the products of 28 enzyme loci for which the electrophoretic mobilities of the mouse and human forms differ. This procedure confirmed the hybrid nature of the clones and determined the extent of their human genome content. A clone was confirmed as a hybrid if it had, for at least one multimeric enzyme: the mouse form, the human form, and the expected number of intermediately migrating hybrid bands consistent with the subunit structure of the enzyme (15). The details of homogenate preparation and vertical starch gel electrophoresis and histochemical stain recipes are contained in ref. 16. The following enzymes were stained for (EC numbers in parentheses): acid phosphatase (3.1.3.2), adenosine deaminase (3.5.4.4), adenylate kinase (2.7.4.3), enolase (4.2.1.11), esterases (3.1.1.–), glucose-6-phosphate dehydrogenase (1.1.1.49), glucosephosphate isomerase (5.3.1.9), glutamate oxaloacetate transaminase (2.6.1.1), glyceraldehyde-3-phosphate dehydrogenase (1.2.1.12), hexoseaminidases (3.2.1.30), isocitrate dehydrogenases (1.1.1.42), lactate dehydrogenases (1.1.1.27), malate dehydrogenases (1.1.1.37), mannosephosphate isomerase (5.3.1.8), nucleoside phosphorylase (2.4.2.1), peptidases (3.4.– –), phosphoglucomutases (2.7.5.1), superoxide dismutase (1.15.1.1), and triosephosphate isomerase (5.3.1.1).

Radiometric ADA Assay. Specific activity of ADA in JEG-3 cells and subclones as well as HeLa cell controls is given in units. Units are defined as μmol adenosine converted to inosine and hypoxanthine per min. This was determined in 100 μl of assay solution containing 5 mM Tris·HCl at pH 7.5, 0.1 mM [^{14}C]-adenosine (Amersham/Searle, Arlington Heights, IL; 49 mCi/mmol), and 0.1–0.01 mg of protein per ml (not to exceed 5% conversion). After 15-min incubation at 37°, the reaction was terminated by plunging the tube into a boiling-water bath. Twenty microliters was spotted on DE-81 DEAE-paper and descending chromatography was conducted to separate metabolites in an isobutyric acid/ammonium hydroxide/water (400:0.4:208 by weight) solvent system. Spots of adenosine, inosine, and hypoxanthine were assayed for radioactivity in a liquid scintillation counter (Packard Instruments, Downers Grove, IL). Protein was determined according to Lowry *et al.* (17).

Chromosomal Analysis. Preliminary chromosomal information (numbers of human chromosome 20 per cell) on JEG-3 and HCC was obtained using Q bands and C bands generated on the same cell according to the method of Chen (18). Quinacrine mustard dihydrochloride produced Q bands and a sequential 65° salt treatment followed by Giemsa staining generated C bands.

***ADA-1-2* Lysates.** Erythrocyte lysates identified as being from *ADA-1-2* heterozygotes were obtained from Robert Ferrell, Genetics Marker Laboratory, Center for Demographic and Population Genetics, University of Texas Health Science Center, Houston, TX.

RESULTS

ADA Expression in Parental Cells and Their Hybrids. Neither HCC nor JEG-3 cells had ADA activity that could be detected on starch gels. A clear, fast-migrating band of ADA activity was readily detectable in both mouse Cl 1D and A9 cells. The mouse form of ADA was expressed in all hybrids. In some hybrids there were two additional bands, in others just the slower and in others just the faster of the two additional bands. Fig. 1*A* also shows that the faster of the two additional bands comigrated with the human form of ADA expressed in HeLa cells. This enzyme in HeLa has been identified as human ADA-1 (20). Therefore, suspecting that these additional bands of activity in the hybrids might represent expression of the products of the human *ADA-1* and *-2* alleles, we ran hybrids expressing both or one or the other of the two extra bands on a gel containing two blood samples obtained from a human *ADA-1-2* heterozygote. As can be seen from Fig. 1*B*, there was perfect comigration between the extra bands in the hybrids and the products of the human *ADA-1* and *-2* alleles. These extra (non-mouse) ADA bands in hybrids will now be referred to as ADA 1 (faster) and ADA 2 (slower).

Table 1. Number of hybrids expressing ADA 1 or 2

Cells hybridized	ADA phenotype 1–2	2	1	0	Total
JEG-3 × Cl 1D	9	10	2	5	26
HCC × Cl 1D	4	1	1	3	9
Subtotal from Cl 1D	13	11	3	8	35
JEG-3 × A9	0	1	0	2	3
HCC × A9	1	1	0	5	7
Subtotal from A9	1	2	0	7	10
Total	14	13	3	15	45

Segregation Analysis of ADA 1 and 2 in Hybrids. The data on the expression of ADA 1 and 2 in the hybrids are contained in Table 1. ADA 1 or 2 became expressed in hybrids formed from all interspecific combinations of parental cells. Fewer hybrid clones produced with A9 (3 out of 10 or 30%) expressed ADA 1 or 2 than hybrids produced with Cl 1D (27 out of 35 or 77%). Examination of the 28 human enzyme locus products studied in each hybrid clone revealed that hybrids with A9 retained fewer human loci on the average (7.0 ± 3.2 or 25%) than hybrids derived with Cl 1D (16.7 ± 4 or 60%).

Higher passages of hybrid clones (which tend to continue to lose human genetic material) either had the same ADA phenotype as an earlier passage or lost a type. A new form was never gained (Table 2). Four hybrid clones derived from HCC or JEG-3 × Cl 1D fusions originally sampled at their 4th, 7th, 8th, and 9th passages had both ADA 1 and 2. When sampled later at their 21st, 21st, 12th, and 26th passages, respectively, they expressed only ADA-2. One hybrid weakly expressing ADA-2 at the 6th passage had only mouse ADA by the 9th. Seven other such hybrids maintained their ADA 1, 2, or 1-2 phenotypes and four consistently produced neither ADA 1 nor ADA 2 when sampled as many as four times up to passages as high as 34. In none of the 16 hybrids sampled through a series of passages was an ADA phenotype, not originally seen in an earlier passage, seen in a later passage.

Table 1 also reveals that a significantly greater number of hybrid clones were ADA 2 than ADA 1 (13 to 3, $\chi^2 = 6.26$, $P < 0.025$). Chromosome analysis of the human lines used revealed that 17 of 25 JEG-3 cells and 14 of 25 HCC cells examined had three copies of chromosome 20. The remainder of the 25 cells studied in both the JEG-3 and HCC lines had two copies

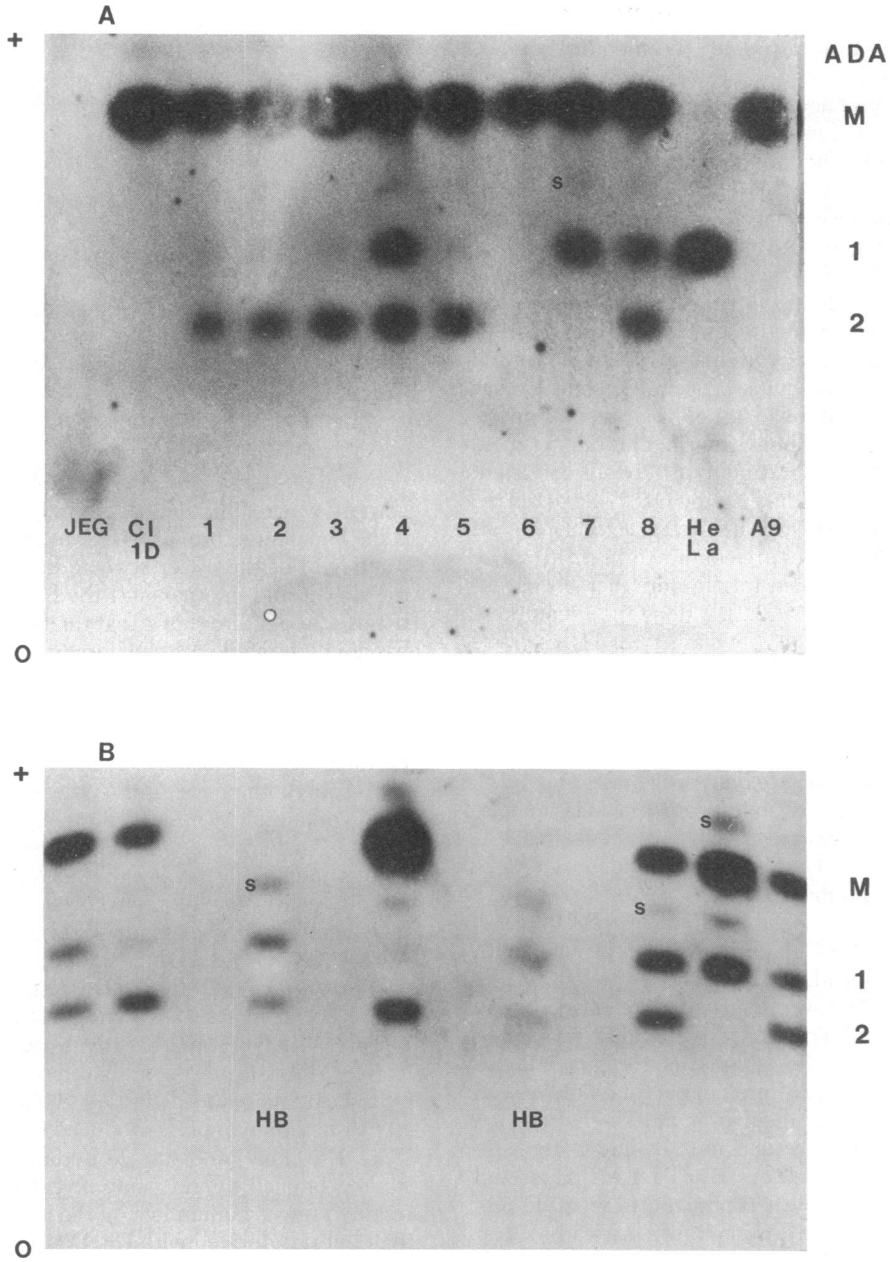

FIG. 1. Starch gel slices histochemically stained for ADA after electrophoresis of cleared cell homogenates. Left-hand margin indicates the origin (O) of the gels and their anodal ends (+). Right-hand margin indicates the migration positions of the mouse form of ADA (M) as well as two additional (non-mouse) forms seen in hybrids and human material. The positions of these latter forms are indicated by 1 and 2. Examples of typical satellites or secondary bands of activity commonly derived from ADA primary bands (19) are indicated as s.

(*A*) Channels containing samples from the parental cells used in the fusions—human JEG, mouse Cl 1D, and mouse A9—are appropriately indicated. A channel containing human HeLa cells, serving as a marker for the position of human ADA 1, is also indicated. The channels marked 1–8 contain samples from eight independently derived JEG-3 × Cl 1D hybrid clones. Extra ADA forms (1 and/or 2) are seen in hybrids but not in the parental cells used for fusion. ADA 1 comigrates with human ADA 1 expressed in HeLa cells. Some hybrids express neither extra band (e.g., no. 6) but continue to produce the mouse form of ADA.

(*B*) Human erythrocyte lysates drawn from *ADA-1-2* heterozygotes are in the channels marked HB. All other samples on the gel are from hybrid clones expressing the faster, the slower, or both of the two extra (non-mouse) bands not seen in either of the parental cell lines used for fusions. These extra bands comigrate with human ADA 1 and 2 from erythrocytes.

of chromosome 20. Chromosome 20 is believed to bear the locus for human ADA (21).

Mixing Experiments. The conditions of hybridization and selection did not induce expression of ADA 1 or 2. Electrophoretic analysis of 27 putative hybrid clones put through the entire fusion and selection procedure revealed them to be human clones. None produced ADA histochemical stain reaction on gels after electrophoresis. Actual hybrid formation appeared necessary, as indicated by the fact that growing JEG-3 and A9 cells together in mixed culture, homogenizing them together, and incubating the mixed homogenates failed to induce production of active ADA 1 or 2.

Homogeneity of JEG-3 Cell Population for ADA Deficiency. Radiometric assay of ADA in JEG-3 revealed a specific activity of 1.6 ± 0.6 units/g of protein (uncertainty is expressed as SEM). This activity was linear with respect to time of incu-

Table 2. ADA 1 or 2 phenotypes expressed in subsequent passages of hybrid clones

Clone	Passages sampled	Respective ADA phenotypes
112b	7 → 21	1-2 → 2
114b-3	9 → 26	1-2 → 2
114c	4 → 16	0 → 0
117c-1	4 → 7 → 21	1-2 → 1-2 → 1-2
119a	7 → 16	0 → 0
122a	8 → 11	1-2 → 1*-2
123b	2 → 5 → 9 → 23	0 → 0 → 0 → 0
124c	6 → 8 → 27 → 34	1 → 1 → 1 → 1
124c-1	7 → 23 → 26	1 → 1 → 1
130e	4 → 21	1-2 → 2
130g	9 → 14	1-2 → 1-2
134b	6 → 24	2 → 2
137a	7 → 12	0 → 0
222a-1	8 → 12	1-2 → 2
222a-2	8 → 19	1-2 → 1-2
222b	6 → 9	2* → 0

* Extremely weak activity, barely visible on gels.

bation and concentration of protein and turned out to be 2.0% the specific activity of HeLa controls (77.8 ± 13.9 units/g of protein). JEG-3 was subcloned to determine whether or not this extremely low level of activity in JEG-3 cells was truly representative of individual cells of the JEG-3 cell population or due to a heterogenous cell population with most of the cells completely devoid of activity and with perhaps 2% of the cells having activity in the range of HeLa. Plating efficiency of subclones was 38%. Six JEG-3 clones were picked and assayed radiometrically for ADA. The specific activities of the six subclones in units/g of protein were 1.51 ± 0.14, 1.89 ± 0.07, 2.69 ± 0.23, 2.76 ± 0.22, 3.01 ± 0.21, and 3.32 ± 0.40. All these subclones failed to produce ADA bands on starch gels after electrophoresis when loaded onto gels at the same protein concentration as the samples in Fig. 1 (~10 mg/ml). An additional 27 independently derived subclones of JEG-3 also failed to produce bands on starch gels following electrophoresis and histochemical staining for ADA.

DISCUSSION

ADA Deficiency in Human Choriocarcinoma Cells. The observation of the lack of expression of ADA 1 or 2 in JEG-3 and HCC on gels has been repeated in our laboratory many times and verified by the low levels observed in the radiometric assays. The radiometric assay of six JEG-3 subclones indicates that the low level of ADA activity detectable in the culture appears to be representative of all cells of the culture and not the result of a mixture of some cells with activity in the HeLa range and most of the cells near zero. This is supported further by the lack of ADA detection on gels in homogenates of those six plus 27 additional JEG-3 subclones.

This deficiency of ADA in our choriocarcinoma cells may be an extension of the differentiated state of the tissue from which they were derived. The possibility of ADA being under tissue-specific regulation was indicated by Lee (22), who demonstrated different levels in different tissues. The choriocarcinoma cells are trophoblastic or fetal in origin (they contain a Y chromosome) and the fetal components of placenta have been shown to contain as little as 1/20 the specific activity of ADA compared to maternal components in mammals as diverse as man, guinea pig, cow, and rat (23–25).

Human ADA 1 and 2 in Hybrid Cells. The additional ADA isozymes in hybrids between our human choriocarcinoma cell lines and mouse fibroblasts we believe to be human ADA 1 and 2. There is no precedent for mouse enzymes producing such clear bands at such a migration point. The bands also migrate too far to be considered the so-called "tissue form" of human ADA (26). The comigration of these isozymes with human ADA 1 and 2 from the *ADA-1-2* heterozygotes as well as the comigration of the faster isozyme in hybrids with ADA 1 from HeLa is strong evidence for this belief.

It has been demonstrated that in human–mouse hybrids, human genetic material became lost or segregated (27). Two aspects of the segregation pattern of the additional ADA isozymes in hybrids are also consistent with the idea that they were the products of human genetic material—the tendency to lose rather than gain an extra isozyme through subsequent passages (Table 2), and the higher frequency of hybrid clones containing the extra isozymes in sets of hybrids which in general retained more human genetic material (fusions with Cl 1D) than in hybrids where human material was not as well retained (A9 fusions).

We therefore conclude that the additional ADA isozymes in the hybrids are human ADA 1 and 2. We attribute the significantly greater frequency of ADA 2 than ADA 1 in hybrids containing only one human type to the uneven number of human chromosomes 20 in a high percentage of human parental cells used in the fusion.

Human ADA Activation by Fusion with Mouse Cells. Fusion of the mouse fibroblasts having ADA with our ADA-deficient human choriocarcinoma cell lines has produced hybrids in which both common allelic forms of human ADA are expressed either together or independently. We have shown this to be highly repeatable (30 out 45 hybrids from four different experiments involving two different parental cell lines from each species) and dependent upon actual fusion. The only barrier to the conclusion that the human gene products specified by information on two separate homologous chromosomes has become activated by presence in the same cell with the mouse genome is the possibility that the mouse cells hybridized with a minor fraction of human cells that already had high ADA activity. Because most hybrids expressed human ADA, the possibility of such an event explaining our results would depend upon a highly preferential fusion of mouse cells with this putative minor fraction. While such a possibility initially seemed remote, it has become a serious consideration in view of the recent report of preferential hybridization of mouse myeloma cells with only the B cells of human peripheral blood lymphocytes used as the human component (28). This finding indicated that an earlier demonstration of human immunoglobin in 10 such hybrids (29) was not due to an induction of human immunoglobin synthesis. In fact, it demonstrated that the mouse cells preferentially (reasons unknown) fused with the minor fraction of blood lymphocytes (40%) that were already differentiated to produce immunoglobin.

Clearly then, in all such fusion experiments in which alterations of a differentiated state of one of the parental components is to be assayed, one must be sure of the homogeneity of that parental component with respect to its state of differentiation for the trait under consideration. In addition to the fact that we used a cloned cell line for 29 of our 45 fusions (in which 22 of the 29 hybrids expressed human ADA), the data reviewed at the beginning of this *Discussion* indicate that the extremely low ADA activity of our choriocarcinoma cell lines used for fusion does not appear to be the result of a mixture of many cells with low activity and a few cells with high activity. Consequently,

our results are not likely to be explained by fusion with the latter type.

We therefore conclude that the hybridization procedure has resulted in the activation of a human gene product. While we cannot yet determine whether the regulatory signal is operating at the transcriptional, processing, translational or post-translational level, we can say that it crosses species lines and simultaneously affects the products of both alleles at the locus. This last point clearly indicates that we are not dealing with a back mutation in a structural locus—a possibility in fusion experiments where the enzyme claimed as being activated was required for survival of hybrids in selective medium (30–34).

Our results extend to human gene products the phenomenon of activation described for rodent genes following cell fusion (35, 36). The high frequency and relatively large number of clones expressing human ADA in our study are much greater than seen for human albumin (37) or hemoglobin (38) expression in mouse–human hybrid clones, so that the possibility of preferential fusion explaining our results is much less likely.

Our finding that the human *ADA* locus is under regulation and that this regulation can be genetically manipulated not only provides an important handle in the study of genetic regulation in human cells, but also presents an alternative conceptual basis (to structural gene variation) for abnormal levels of ADA associated with severe combined immunodeficiency and inherited hemolytic anemia.

We thank Marilyn Buck, Billie White, and Jeffrey Greenspan for their excellent technical assistance. Discussions with Dr. David A. Wright of M. D. Anderson Hospital and Tumor Institute were particularly helpful during the course of this work. We gratefully acknowledge the support of National Institutes of Health Grants ES 02187, AM 21458, GM 19513, and CA 01935. P.O.K. is an Investigator of the Howard Hughes Institute.

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