Adenosine Deaminase Messenger RNAs in Lymphoblast Cell Lines Derived from Leukemic Patients and Patients with Hereditary Adenosine Deaminase Deficiency

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ABSTRACT Hereditary deficiency of adenosine deaminase (ADA) usually causes profound lymphopenia with severe combined immunodeficiency disease. Cells from patients with ADA deficiency contain less than normal, and sometimes undetectable, amounts of ADA catalytic activity and ADA protein. The molecular defects responsible for hereditary ADA deficiency are poorly understood. ADA messenger RNAs and their translation products have been characterized in seven human lymphoblast cell lines derived as follows: GM-130, GM-131, and GM-2184 from normal adults; GM-3043 from a partially ADA deficient, immunocompetent !Kung tribesman; GM-2606 from an ADA deficient, immunodeficient child; CCRF-CEM and HPB-ALL from leukemic children. ADA messenger (m)RNA was present in all lines and was polyadenylated. The ADA synthesized by in vitro translation of mRNA from each line reacted with antisera to normal human ADA and was of normal molecular size. There was no evidence that posttranslational processing of ADA occurred in normal, leukemic, or mutant lymphoblast lines. Relative levels of specific translatable mRNA paralleled levels of ADA protein in extracts of the three normal and two leukemic lines. However, unexpectedly high levels of ADA specific, translatable mRNA were found in the mutant GM-2606 and GM-3043 lines, amounting to three to four times those of the three normal lines. Differences in the amounts of ADA mRNA and rates of ADA synthesis appear to be of primary importance in maintaining the differences in ADA levels among lymphoblast lines with structurally normal ADA. ADA deficiency in at least two mutant cell lines is not caused by deficient levels of translatable mRNA, and unless there is some translational control of this mRNA, the characteristic cellular ADA deficiency is most likely secondary to synthesis and rapid degradation of a defective ADA protein.

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INTRODUCTION

The enzyme adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4)[1] catalyzes the irreversible deamination of adenosine and deoxyadenosine. Special attention was drawn to this enzyme in 1972 when Giblett et al. (1) reported two young unrelated girls with severe combined immunodeficiency disease who also had absence of ADA activity in their erythrocytes. Since that time there has been intensive probing of the relationship between ADA and immune function. ADA deficiency causes abnormalities of deoxynucleotide and S-adenosylhomocysteine metabolism with secondary toxicity to lymphocytes and lymphoid precursors. The metabolic consequences of ADA deficiency have been thoroughly reviewed by Thompson and Seegmiller (2) and Martin and Gelfand (3).

Less is known about the molecular basis of human ADA deficiency than about its metabolic consequences. A number of theories have been proposed to explain the loss of ADA activity in hereditary ADA deficiency. Possible explanations include (*a*) a mutation in the ADA structural gene (1, 4), (*b*) a mutation in a gene regulating ADA expression (5), (*c*) a defect

[1] *Abbreviations used in this paper:* ADA, adenosine deaminase; mRNA, messenger RNA; poly(A)$^+$RNA, RNA that binds to oligo(dT) cellulose in high-salt (0.5 M NaCl) buffer; poly(A)$^-$RNA, RNA that does not bind to oligo(dT) cellulose in high-salt buffer.

in posttranslational modification of the ADA protein (6), and (*d*) a chromosomal deletion in the region of the ADA gene (1). In recent work from our laboratory (7) ADA was examined in lymphoblast cell lines established from normal and ADA-deficient individuals. ADA activity was determined by enzymatic assay and compared to ADA protein determined by two different radioimmunoassays. Using this approach, ADA-deficient cells could be divided into three types, representing at least three different mutations. It was not known whether cells were homozygous for one or heterozygous for two different mutant ADA alleles. The first type of mutant cell had 33–50% of normal enzyme activity and 50–100% of normal amounts of ADA protein. The ratio of protein to enzyme activity was twice normal. These cells undoubtedly carry a mutation in the structural gene for ADA that codes for an unstable and/or catalytically defective protein. To date, all individuals with this type of mutation have been immunocompetent. A second type of mutant cell had both very low enzyme activity and very low ADA protein, but a normal ratio of protein to activity. In this case reduced levels of structurally normal ADA could have been synthesized or, alternatively, a structurally defective protein could have lost catalytic and antigenic activity in a coordinate manner. A mutation might have occurred in either a regulatory or a structural region of the ADA gene. The third type of mutant cell was indistinguishable from the second type when rabbit antiserum was used to measure ADA protein in the radioimmunoassay. However, when goat antiserum was used, ADA protein equivalent to 12% of normal was detected. This third type of cell could carry a mutation in the ADA structural gene, different from the first type, that results in synthesis of catalytically inactive, very unstable protein. A wide range in the ratio of ADA protein to catalytic activity has also been demonstrated in ADA-deficient erythrocytes and fibroblasts by Carson et al. (8) and Daddona et al. (9, 10). Their studies are also consistent with the hypothesis that several different mutations that cause ADA deficiency have occurred in humans.

Hypotheses about the molecular basis of ADA deficiency can be derived from immunological studies of ADA protein and measurements of enzymatic activity. Choosing among the hypotheses is difficult, however, because of the apparent lack of ADA protein in some mutant cells, and the small amounts of ADA protein in most other cells. This lack of ADA protein makes proof of normal or abnormal amino acid sequence difficult or impossible. Considerable additional information about these mutations and the normal synthesis of ADA can be obtained by studies of ADA messenger (m)RNAs and their translation products from normal and mutant cells. We have isolated mRNAs from lymphoblast cell lines derived from normal individuals and individuals with the first and third types of ADA deficiency described above, GM-3043 and GM-2606 cell lines, respectively. Comparison of the amounts and types of ADA proteins synthesized by in vitro translation of these RNAs has permitted further testing of hypotheses about the nature of genetic mutations causing human ADA deficiency.

METHODS

Materials

Rabbit reticulocyte-rich whole blood was purchased from Pel-Freeze Biologicals (Rogers, AR). Sources of other materials included: ribonuclease-free urea and sodium deoxycholate, Schwarz/Mann, Inc., Spring Valley, NY; vanadyl sulfate, Fisher Scientific Co., Fair Lawn, NJ; micrococcal nuclease, P-L Biochemicals, Inc., Milwaukee, WI; creatine phosphate, creatine phosphokinase, sarkosyl (*N*-lauroyl sarkosine), Triton N-101, and phenylmethylsulfonyl fluoride, Sigma Chemical Co., St. Louis, MO; oligo(dT)-cellulose, type III, Collaborative Research, Inc., Waltham, MA; [^{35}S]methionine (>600 Ci/mmol), Amersham Corp., Arlington Heights, IL; Whatman GF/C paper, Curtin Matheson Scientific, Inc., Cincinnati, OH; IgGsorb brand of protein A containing *Staphylococcus aureus*, The Enzyme Center, Boston, MA; En^{3}Hance and Protosol, New England Nuclear, Boston, MA; guanidine thiocyanate, Eastman Kodak Co., Rochester, NY. Vanadyl complexes of guanosine, adenosine, cytidine, and uridine were prepared according to the method of Berger and Birkenmeier (11).

Cell lines

The lymphoblast cell lines GM-130, GM-131, GM-2184, GM-3043, and GM-2606 are transformed B cell lines that were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). GM-130, GM-131, and GM-2184 were established from people with normal ADA activity, whereas both GM-3043 and GM-2606 are from ADA-deficient humans. The CCRF-CEM cells were obtained from the American Type Culture Collection (Rockville, MD). The HPB-ALL cells were a gift from Dr. Jun Minowada, Roswell Park Memorial Institute (Buffalo, NY). Both CCRF-CEM and HPB-ALL are leukemic lymphoblasts established from children with acute lymphoblastic leukemia and have structurally normal ADA. Cells were grown in RPMI 1640 medium supplemented with 10–20% fetal calf serum. To confirm the identity of each cell line, they were typed for surface markers using a variety of monoclonal reagents. The OKT3 and OKT4 antibodies (Ortho Diagnostic Systems, Inc., Raritan, NJ) react with certain peripheral T lymphocytes and their precursors. The BI and I2 antibodies (Coulter Electronics, Hialeah, FL) react with certain peripheral B lymphocytes and their precursors. The CCRF-CEM cells and HPB-ALL cells were verified to be T lymphoblasts (OKT3$^-$, OKT4$^+$, BI$^-$, I2$^-$ and OKT3$^+$, OKT4$^+$, BI$^-$, I2$^-$, respectively). The other lines were B lymphoblasts (OKT3$^-$, OKT4$^-$, BI$^+$, I2$^+$).

Antisera

Rabbits and goats were immunized with pure homogeneous human ADA prepared from leukemic cells collected

by leukophoresis (12). The antisera to native ADA have been described and have been used extensively in radioimmunoassay of normal and mutant ADA (7). Additional rabbit antisera were prepared against sodium dodecyl sulfate (SDS) denatured homogeneous human ADA using the immunization schedule previously described (7). All antisera gave single bands when diffused against either the antigen used for immunization or extracts of leukemic lymphoblasts.

Extraction of total RNA

Total RNA was extracted from 3–10-g wet weight of late log phase cells by one of the following three methods. The concentration of RNA solutions was determined spectrophotometrically; 1.0 OD_{260} unit was estimated to be equivalent to 50 μg/ml RNA.

Method I. This is a minor modification of the method of Coleman and Riley.[2] Cells were harvested from culture medium by centrifugation at 3,100 *g* for 5 min. The fresh cell pellet or cells that had been frozen in liquid nitrogen were mixed with or dropped into cold (4°C) extraction buffer (100 ml of buffer to 5 g of cells) and immediately blended at highest speed for 1 min in a Waring blender. The RNA extraction buffer was 10 mM sodium acetate, pH 5.0, 3 M LiCl, 0.1% SDS, 6 M urea (ribonuclease-free), 2 mg/ml heparin, and 6 mM vanadyl ribonucleoside complexes. The homogenate was placed at −20°C overnight. The precipitated RNA was then isolated by centrifugation at 10,000 *g*, 25 min, 4°C. The pellet from 5 g of cells was dissolved in 12 ml of autoclaved water and extracted twice with equal volumes of phenol/chloroform (1:1, vol/vol) and then once with 12 ml of chloroform. The phenol had been saturated with 0.3 M sodium acetate, pH 5.0, 0.3% SDS, and was mixed with the RNA solution before the addition of chloroform to prevent protein-RNA aggregation (13). The extracted RNA was then precipitated at −20°C overnight following the addition of one-tenth volume of 3 M sodium acetate, pH 6.0, and 2.5 vol of cold absolute ethanol. The precipitated RNA was recovered by centrifugation at 25,000 *g*, 30 min, −20°C. The RNA pellet was washed once or twice with cold 3 M sodium acetate, pH 6.0 (1 ml to ~10 mg RNA) and one time with cold (−20°C) 70% ethanol. The washes removed the greenish vanadyl ribonucleoside complexes that inhibit in vitro translation. The washed RNA was then dissolved in autoclaved water and reprecipitated from ethanol as before. This procedure yielded about 2 mg of RNA/g of cells.

Method II. The procedure was described in detail by Berger and Birkenmeier (11) for isolation of RNA from the cytoplasm of resting lymphocytes. Cells are lysed in a low salt buffer containing Triton N101 (1.2%) and, as a ribonuclease inhibitor, vanadyl ribonucleoside complexes (10 mM). The nuclei are removed by centrifugation and the supernatant fraction is extracted with phenol and chloroform. The RNA is precipitated with ethanol and washed as described for Method I.

Method III. This is a minor modification of the method of Chirgwin et al. (14). Freshly harvested lymphoblasts, 1 g, were suspended in 16 ml of a buffer containing 4 M guanidine thiocyanate, 0.2% sarkosyl, 25 mM trisodium citrate, pH 7, 200 mM β-mercaptoethanol and homogenized in a glass homogenizer for ~18 strokes. The homogenization buffer was prepared without the β-mercaptoethanol, filtered to remove insoluble material, and autoclaved. The β-mercaptoethanol was added immediately before use. Cellulose nitrate tubes were freed of ribonuclease by immersion in boiling 100 mM Na_2EDTA for 5 min followed by rinsing twice with autoclaved water. The homogenate was layered over an equal volume of autoclaved 5.7 M CsCl, 100 mM EDTA, pH 7, in the boiled tubes. They were centrifuged at 100,000 *g* for 19 h at 20°C. Following centrifugation, the guanidine thiocyanate layer was removed. The sides of the tube were rinsed with 2–3 ml of fresh guanidine thiocyanate solution. This and most of the CsCl layer were removed. The sides of the tube above the residual CsCl were cut away using scissors and any remaining CsCl was decanted. A small opalescent pellet of RNA remained in the bottom of the tube. The RNA was washed in a small amount of 70% ethanol and then dissolved in 10 mM Tris HCl, pH 7.4, 1.0 mM EDTA, and precipitated from ethanol. The RNA preparations were redissolved in autoclaved water or 10 mM Tris HCl, pH 7.4, 1.0 mM EDTA, and stored in liquid nitrogen.

Isolation of polyadenylated mRNA

Total RNA was fractionated by chromatography on oligo(dT)-cellulose (15). The oligo(dT)-cellulose (0.5–1.0 g in a 0.7 × 10-cm column) was washed with 0.1 M KOH and then neutralized with 1 M Tris HCl, pH 7.4. Before use it was equilibrated with 50–60 ml of high salt buffer (10 mM Tris HCl, pH 7.4, 1.0 mM EDTA, 0.5 M NaCl). The RNA solution (1 mg RNA/ml in 10 mM Tris HCl, pH 7.4, 1 mM EDTA) was denatured by heating 5 min at 75°C, rapidly cooled, and then made 0.5 M in NaCl by addition of 5 M NaCl before loading on the column. Poly(A)$^-$RNA was eluted with high salt buffer, ~30 ml. The poly(A)$^+$RNA was then eluted with 10 mM Tris HCl, pH 7.4, 1 mM EDTA. Fractions (1 ml) containing 0.1 OD_{260} units or more of RNA from this low salt elution were pooled, made 0.5 M in NaCl, and chromatographed in the same manner a second time. The poly(A)$^+$RNA from the second low salt elution was precipitated from ethanol and stored as described above for total RNA. In some cases 0.2% SDS was included in the high salt buffer and the poly(A)$^+$RNA was eluted only once with low salt buffer. The poly(A)$^+$RNA obtained by this latter method was quite comparable to the first in stimulation of protein synthesis in vitro and in relative content of ADA mRNA. The final yield of poly(A)$^+$RNA was ~3% of the starting RNA.

In vitro translation

In early experiments, RNA translations were performed using in vitro translation kits obtained from New England Nuclear Corporation. In later experiments lysates were prepared from reticulocyte-rich whole rabbit blood as described by Comstock et al. (16) and made mRNA dependent by treatment with 10 μg micrococcal nuclease/ml for 10 min (17). Translation assays contained, in a total volume of 25 μl, ~50 μCi of [^{35}S]methionine (~1,000 Ci/mmol), 0.5 μg creatine phosphokinase, 10 mM creatine phosphate, 2 mM dithiothreitol, 80 mM potassium acetate, 0.6 mM magnesium acetate, 10 μl of lysate, and RNA. Incubations were at 37°C. Reactions were monitored by spotting aliquots on 2 × 2-cm squares of GF/C paper and immediately dropping them into cold 10% trichloroacetic acid (TCA). The squares were washed once for 10 min with cold 10% TCA, twice for 10 min each with boiling 10% TCA, and twice, for 2 min each, with ethanol. The dried squares were counted in a toluene-based scintillation cocktail using a Beckman LS 7000 counter (Beckman Instruments, Inc., Fullerton, CA).

[2] Coleman, M. S., and L. Riley. Personal communication.

Immunoprecipitation of ADA synthesized by in vitro translation

In vitro translation reactions to be used for immunoprecipitation of ADA were scaled up to 80–300 μl and were allowed to proceed for 45–55 min. After this time, 2-μl aliquots were spotted on GF/C squares and processed as described above. The remaining in vitro translation product, i.e., the complete in vitro translation reaction mixture after incubation, was diluted with 2.5 vol of immunoprecipitation buffer containing 1 mM phenylmethylsulfonyl fluoride. The immunoprecipitation buffer was 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 M NaCl, 1 mM Na_2EDTA, 1 mM methionine, and 10 mM sodium phosphate, pH 7 (18). Following the addition of 1 μl of nonimmune serum, the diluted in vitro translation product was incubated at 4°C for 1 h. Then, 100 μl of 10% IgGsorb in immunoprecipitation buffer was added and the mixture was incubated 15 min at room temperature. The IgGsorb and some nonspecifically bound proteins were removed by centrifugation at 15,000 *g* for 5 min. Anti-ADA serum, or nonimmune serum, as a control, was then added to the supernatant, and this mixture was incubated for 1 h at 4°C. The immune complex was precipitated by addition of 100 μl of 10% IgGsorb followed by a 15-min incubation at room temperature and centrifugation for 10 s at 15,000 *g*. The pellet was washed three times with 0.5 ml of immunoprecipitation buffer and then dissociated by a 1-h, room temperature incubation in 70 μl of 10 mM Tris HCl, 1% SDS, 8 M urea, and 1 mM phenylmethylsulfonyl fluoride. The IgGsorb was removed by centrifugation and the dissociated, supernatant proteins were then fractionated by electrophoresis on 10% polyacrylamide, SDS-containing slab gels, 1.5 mm thick (19). The radioactive protein bands were visualized by fluorography according to the method of Bonner and Laskey (20). Gels were treated with En^{3}Hance and exposed to x-ray film using an intensifying screen. Visualization required 4–18 d of exposure depending on the amount of [^{35}S]methionine that was incorporated into the in vitro translation product.

Quantitation of ADA protein synthesized in vitro

The ADA synthesized in vitro was labeled with [^{35}S]-methionine and therefore could be quantitated by determining the radioactivity in the gel slices that corresponded to the area to which standard ADA migrated. When wet gels were to be sliced and counted, purified ADA, 1.0 μg, was often added to the samples of extracted immunoprecipitated protein before electrophoresis. The purified ADA was located by staining gels with Coomassie Blue. Slices were taken that corresponded to the stained ADA. Because the Coomassie Blue stain quenched fluorography, to prepare autoradiographs purified ADA was electrophoresed and stained in an adjacent lane instead of the lane containing radioactive protein. The radioactivity in ADA protein was determined by subtracting the counts in gel slices from areas with the R_f of ADA in nonimmune serum-treated in vitro translation product from the counts in the gel slices from areas containing ADA in immune serum-treated in vitro translation product. In each case an equal volume from the same in vitro translation reaction was mixed with either immune or nonimmune sera. To determine the radioactivity in the gel slices, 2-mm slices of a gel were incubated at 37°C overnight in 8 ml of 5% Protosol in a toluene-based scintillation fluid. Samples were then counted in a Beckman LS 7000 counter.

Agarose gels

Samples of RNA, 25 μg, were denatured in 1.0 M glyoxal and fractionated on 1.5% agarose slab gels as described by McMaster and Carmichael (21).

Assay of ADA enzymatic activity

ADA activity was assayed with [^{14}C]adenosine as the substrate (7). Samples were assayed with and without the specific ADA inhibitor, erythro-9-(2-hydroxy-3-nonyl)adenine (0.35 mM), to distinguish ADA from a second aminohydrolase that is present at low levels and not sensitive to this inhibitor. The microbiuret method was used for protein determinations (22).

RESULTS

Isolation and in vitro translation of RNA from lymphoblast cell lines. Because lymphoblasts contain relatively high levels of ribonuclease activity (11), the procedures for isolating intact mRNA from these cells must incorporate techniques, inhibitors, and denaturing agents that minimize the activity of these degradative enzymes. The quality of a preparation of RNA can be assessed by measuring both its ability to stimulate in vitro protein synthesis and its size distribution on agarose gels. Low average molecular weight and poor stimulation of in vitro protein synthesis indicate degradation. RNA was isolated from CCRF-CEM lymphoblasts by three different methods (Table 1). The yield of RNA ranged from 1.5 to 2.7 mg/g wet weight of cells. Electrophoresis on agarose gels of 25 μg of RNA prepared by each method showed that the RNA isolated from the cytoplasm by method II contained less of the highest molecular weight RNAs than RNA isolated by methods I and III. The loss of high-molecular weight species indicated partial degradation of RNA, when prepared by method II. Preparations of RNA were translated in nuclease-treated reticulocyte lysates (Table I). The RNA isolated by method I stimulated more incorporation of [^{35}S]methionine into protein per unit weight than RNAs isolated by methods II and III. However, equal amounts of poly(A)$^+$RNA prepared from RNAs isolated by methods I and III stimulated protein synthesis equally well, so both methods are probably satisfactory when applied to fresh cells. Method I could also be readily applied to cells frozen in liquid nitrogen and was adopted for general use in our laboratory.

Poly(A)$^+$RNA was prepared by oligo(dT)-cellulose chromatography of total RNA from lymphoblasts. In vitro translation of the poly(A)$^+$RNA gave a three- to sevenfold stimulation of protein synthesis over endog-

TABLE I
In Vitro Translation of RNAs Prepared by Three Different Methods

RNA*	Yield of RNA from CCRF-CEM cells	Protein synthesized in vitro: Radioactivity in total protein	Protein synthesized in vitro: Fold stimulation over endogenous protein synthesis‡
	mg/g wet wt of cells	*cpm*	
None added		27,730	
Prepared by method I			
Batch 1	2.7	77,910	2.8
Batch 2	1.8	76,750	2.8
Prepared by method II	1.9	44,580	1.6
Prepared by method III	1.5	40,510	1.5

* CCRF-CEM cells were grown to a density of 2×10^6 cells/ml and then harvested by centrifugation. RNA was isolated by three different methods. Each RNA was translated in nuclease-treated reticulocyte lysates with [^{35}S]methionine as the radioactive amino acid. The reaction volume was 25 μl, concentration of total RNA was 350 μg/ml, and reaction time was 45 min.

‡ Calculated as the ratio of counts per minute incorporated in the presence of exogenous RNA from CCRF-CEM to counts per minute incorporated in the absence of exogenous RNA.

enous levels. Globin mRNA obtained from New England Nuclear Corporation gave three- to sixfold increases over endogenous levels of protein synthesis in the same systems. The optimal concentration of added magnesium acetate for synthesis of total protein using lymphoblast RNA and reticulocyte lysates prepared in our laboratory was 0.6 mM. The relationship between the amount of poly(A)$^+$RNA added to a lysate and the amount of total protein synthesized was linear from 2 to ~20 μg RNA/ml as shown in Fig. 1.

Demonstration of the presence of ADA mRNA in poly(A)$^+$RNA. Human ADA was purified by affinity chromatography to homogeneity (12). This pure homogeneous enzyme was used to immunize animals and to compete with translation products for antibody binding sites. Three different antisera to ADA were used to immunoprecipitate newly synthesized ADA protein from the total in vitro translation products, i.e., the resultant total in vitro translation mixture following incubation at 37°C. Fluorography of 10% polyacrylamide-SDS gels containing the electrophoresed, immunoprecipitated protein revealed a band that migrated to the position of authentic human ADA, molecular weight 44,000 (Fig. 2, lane B vs. A). This band was present in in vitro translation products immunoprecipitated with rabbit antisera raised to both native and SDS-denatured ADA and with goat antiserum to native ADA. The band was not demonstrated in in vitro translation products treated with nonimmune serum from the same rabbits that furnished the antisera against native ADA (Fig. 2). Although the bands that migrated as authentic ADA were obvious in this gel, a number of other bands were also present. These bands represent nonimmunospecific binding, mostly

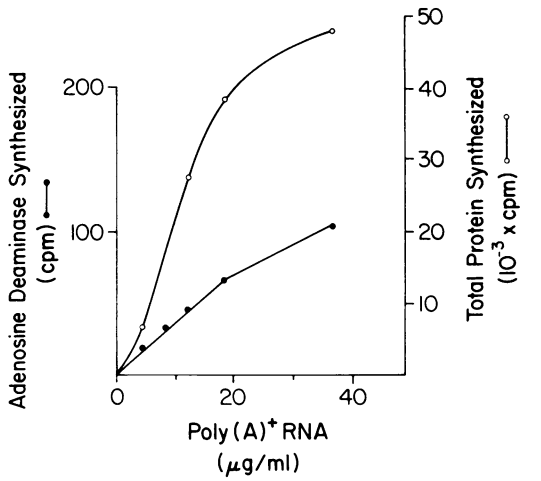

FIGURE 1 Relationship between in vitro synthesis of immunoreactive ADA and the concentration of poly(A)$^+$RNA in the translation mixture. In vitro translations of poly(A)$^+$RNA from CCRF-CEM cells were performed as described in the Methods with 80 μl reaction volumes and [^{35}S]methionine as the tracer amino acid. Reaction time was 45 min. Radioactivity in the total protein was determined from a 2-μl aliquot. ADA in the remaining 78 μl of the reaction was immunoprecipitated and electrophoresed on 10% polyacrylamide-SDS gels. The radioactivity of ADA was determined in slices of the dried gel that corresponded to the location of standard purified human ADA.

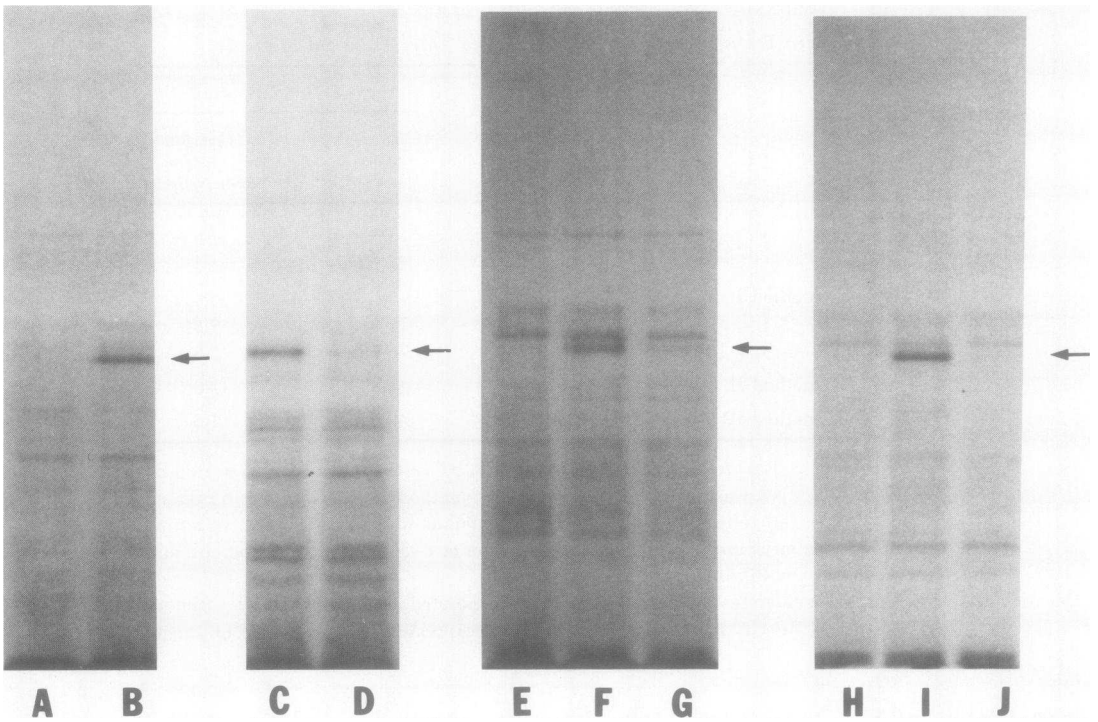

FIGURE 2 Demonstration of ADA-specific mRNA in poly(A)$^+$RNA from CCRF-CEM, GM-2606, and GM-3043 cells. The photographs show autoradiographs of polyacrylamide-SDS gels after electrophoresis of radiolabeled, immunoprecipitated protein from in vitro translations of poly(A)$^+$RNA. Proteins were precipitated using antiserum against native human ADA. Lanes A through D were obtained from 100 μl reactions containing CCRF-CEM poly(A)$^+$RNA (39 μg/ml); lanes E through G were obtained from 150 μl reactions containing GM-2606 poly(A)$^+$RNA (20 μg/ml). Lanes H through J were obtained from 100 μl reactions containing GM-3043 poly(A)$^+$RNA (20 μg/ml). Translation was carried out for 50 min. The arrows mark the position of migration of purified human ADA. Lane A. Rabbit nonimmune serum, 3 μl, was incubated with the in vitro translation products before precipitation with IgGsorb. Lane B. Rabbit antiserum directed against native human ADA, 3 μl, was incubated with the in vitro translation products before precipitation with IgGsorb. Lane C. The same treatment as lane B except 1 μl of antiserum was used. Lane D. Same as lane C except purified human ADA, 2.3 μg, was added to the in vitro translation products before addition of 1 μl of antiserum. Lane E and H. Rabbit nonimmune serum, 1 μl, was incubated with the in vitro translation product before precipitation with IgGsorb. Lane F and I. The same as lane E except 1 μl of antiserum was used instead of nonimmune serum. Lane G. The same as lane F except purified human ADA, 5.8 μg, was added to the in vitro translation product before the addition of 1 μl antiserum. Lane J. The same as lane I except purified human ADA, 11.6 μg, was added to the in vitro translation product before the addition of 1 μl antiserum.

to the IgGsorb, of some of the proteins that were synthesized in much larger amounts than ADA in the in vitro system. ADA comprises 0.01% or less of protein synthesized in vitro. Additional evidence that the band migrating in the same position as ADA was actually ADA was sought by competition experiments. The ADA used in these competition experiments had been purified to homogeneity by adenosine affinity chromatography. It migrated as a single band on SDS polyacrylamide-gel electrophoresis, nondenaturing polyacrylamide gel electrophoresis (44,000 mol wt), and isoelectric focusing (12). When the purified human ADA was added to the in vitro translation products before immunoprecipitation with a limiting amount of antiserum to ADA, the intensity of the ADA band was progressively reduced with the addition of 2.3 and 5.8 μg of ADA (Fig. 2, lane C vs. D and lane F vs. G) and became negligible with the addition of 11.6 μg of ADA (Fig. 2, lane I vs. J). The direct competition between authentic homogeneous ADA and the translation product for specific anti-ADA antibody binding sites confirms identification of the immunoprecipi-

tated in vitro translation product as ADA. The existence of newly synthesized ADA proves the presence of ADA mRNA in the poly(A)$^+$RNA preparation from lymphoblasts.

Characterization of ADA mRNAs and their translation products in normal, leukemic, and mutant lymphoblast cell lines. ADA was demonstrable in specific immunoprecipitations of in vitro translation products from poly(A)$^+$RNAs, but not from poly(A)$^-$RNAs. Therefore, the ADA messenger is polyadenylated.

The primary goal of this project was to compare in vitro translation products of ADA mRNAs from normal and ADA-deficient lymphoblast cell lines both by relative amounts and by molecular size of the ADA synthesized. If amounts of ADA synthesized in vitro by different sources of RNA are to be compared quantitatively, the amounts of antiserum used must be sufficient to precipitate the maximum amount of the relevant antigen present in the translation mixture. In the experiments shown in lanes C through J of Fig. 2, the antiserum was limited to show competition between the newly synthesized radiolabeled ADA and the authentic unlabeled ADA. We have experimentally demonstrated that 3 μl of our rabbit antiserum to native ADA are sufficient to bind the amount of ADA synthesized in 60 min from 4.0 μg of poly(A)$^+$RNA from HPB-ALL or CCRF-CEM cells. These two cell lines contain the highest levels of ADA catalytic activity and presumably ADA mRNA of all the lines we have studied. Using 3 μl of antiserum for immunoprecipitation of in vitro translation products, the amount of ADA protein synthesized increased linearly with increased amounts of poly(A)$^+$RNA added to the translation reactions (Fig. 1).

Poly(A)$^+$RNA prepared from seven different lymphoblast cell lines was translated in vitro, and the newly synthesized radiolabeled ADA was immunoprecipitated and electrophoresed on polyacrylamide-SDS gels as described in Methods. The radioactivity in ADA proteins was determined in appropriate gel slices. The amounts of total protein and ADA protein synthesized in vitro using poly(A)$^+$RNAs from the seven cell lines are shown in Table II. The values in columns two and three are the averages of the counts per minute determined in all experiments using the specified poly(A)$^+$RNA concentrations. The percentage of total incorporation that was in ADA and the number of separate experiments with RNA from each cell line are listed in column 4. Relative incorporation into ADA is a measure of the relative amount of ADA-specific mRNA compared with the total amount of translatable mRNA. Relative amounts of ADA synthesized were invariably greatest in the leukemic T lymphoblast RNA translation products (0.011–0.015%) and least in translation products of RNA from the normal B cell lines (0.0014–0.0023%). The relative synthesis of ADA in vitro paralleled the relative levels of enzyme catalytic activity and immunoreactive protein found in these cell lines in vivo (Table II, columns 5–7). Rather unexpectedly, translations of poly(A)$^+$RNA from the two ADA-deficient B cell lines consistently yielded three to four times greater relative amounts of ADA than did RNA from the normal B cell lines (Table II, columns 4, 5). In cases shown in Table II, where only two determinations of relative amounts of ADA were made the greatest variation of the extremes from the mean was 17%. For example, the two values determined for in vitro synthesis of ADA from GM-3043 poly(A)$^+$RNA (20 μg/ml) were 0.0056 and 0.0072%, a variation of 12% from the mean. To determine consistency in percent of in vitro synthesized protein that was ADA from different preparations of cells, poly(A)$^+$RNA was prepared from two batches of CCRF-CEM cells and two batches of GM-2184 cells. The two batches of poly(A)$^+$RNA from GM-2184 cells gave percentages of ADA synthesized in vitro of 0.0018 and 0.0025, those from CCRF-CEM gave 0.0087 and 0.0110, respectively.

Most determinations of percentages of ADA synthesized were from reactions containing 20 μg poly(A)$^+$RNA/ml, because up to this RNA concentration both total protein synthesis and ADA synthesis increased linearly (Fig. 1). However, the relative amounts of ADA synthesized were also quite comparable at 40 μg RNA/ml (Table II).

Relatively high levels of total protein were synthesized by in vitro translation programmed by GM-2184 poly(A)$^+$RNA, 20 μg/ml, but the percentage of ADA synthesized (0.0018±0.0007) was low, compared with ADA-deficient and leukemic T lines (Table II). To determine if there might be some type of inhibitor of ADA messenger translation in these GM-2184 preparations, one in vitro translation was programmed with a one-to-one mixture of GM-2184 and CCRF-CEM poly(A)$^+$RNA (20 μg RNA/ml). The percentage of ADA synthesized in this reaction, 0.006, was intermediate between the ADA synthesized by the two reactions individually. Therefore, there was no specific inhibitor of translation of ADA mRNA in the GM-2184 poly(A)$^+$ preparation. To determine whether the low relative content of ADA-specific mRNA was unique to the GM-2184 cell line, two additional normal B lines were studied. Both GM-130 and GM-131 were similar to GM-2184 in relative ADA mRNA content, so the property is not unique (Table II).

The size of ADA proteins was determined by comparison of migrations of the immunoprecipitated newly synthesized ADA with migration of authentic human ADA (12) and other standard proteins on polyacrylamide-SDS gels. The poly(A)$^+$RNAs from all

TABLE II

Comparative Amounts of Translatable ADA-specific mRNA, ADA Catalytic Activity, and ADA-specific Immunoreactive Protein in Normal and ADA-deficient Cell Lines

Source of the poly(A)⁺RNA	Concentration of poly(A)⁺RNA in the translation reaction (1)	In vitro protein synthesized*			Relative ADA‡		
		Total (2)	ADA specific (3)	ADA/total (4)	mRNA content (5)	Catalytic activity (6)	Immunoreactive protein (7)
	μg/ml	*cpm × 10⁻³*	*cpm*	*percent × 10²*			
Normal B lines							
GM-130	20	2,330	33	0.14 (1)§	0.8	0.8	
GM-131	20	2,690	62	0.23 (1)	1.3	0.8	
GM-2184	20	3,420	64	0.18±0.07 (4)	1.0	1.0	1.0
	40	4,950	69	0.14 (1)			
ADA-deficient B line							
GM-2606	20	4,260	319	0.73±0.2 (3)	4.1	0.01	0.12
Partially ADA-deficient B line							
GM-3043	20	2,360	154	0.64 (2)	3.6	1.8	1.07
	40	2,470	163	0.66 (1)			
Leukemic T lines							
CCRF-CEM	20	2,380	261	1.1±0.2 (5)	6.1	7.1	7.1
HPB-ALL	20	1,350	204	1.5 (2)	8.3	6.8	
	40	1,780	242	1.4 (2)			

* Measured as counts per minute [^{35}S]methionine incorporated per 100 μl reaction mixture. In vitro translations of poly(A)⁺RNA were performed in the following volumes: GM-130, GM-131, GM-2184 and GM-3043, 300 μl; GM-2606, 150 and 300 μl; CCRF-CEM and HPB-ALL, 100 μl. Reaction times were 50 min.

‡ Relative values were calculated by setting the value for the normal B line, GM-2184, as 1.0. Relative mRNA content is based on translation assays containing 20 μg poly(A)⁺RNA/ml. Catalytic activities were by measurement of ADA activity in extracts of the same batch of cells used to prepare RNA except for HPB-ALL cells where the original cells were not assayed and a second batch was tested. ADA activity in GM-2184 was 53 nmol inosine/min per mg protein. Immunoreactive ADA protein was measured by radioimmunoassay using goat antiserum to human ADA (GM-2184, GM-2606, CCRF-CEM, HPB-ALL) or rabbit antiserum to human ADA (GM-2184, GM-3043) (see reference 7 for a discussion of the properties of these sera when used to assay mutant proteins). The values reported for GM-3043 and GM-2606 represent the greatest amount of protein detected by any type of radioimmunoassay. GM-2184 cells contained 645±161 (n = 5) ng/10^8 cells using goat antiserum and 800 ng/10^8 cells using rabbit antiserum. Catalytic activities and immunoreactive protein were not measured in the same batches of cells.

§ Number of separate determinations that were averaged is shown in parentheses. The ratio, ADA/total, for CCRF-CEM, GM-2184, and GM-2606 shows the mean±SD. Values for GM-2184 and CCRF-CEM each include determinations made on RNAs isolated from two batches of cells.

seven of the lymphoblast cell lines yielded ADA proteins that were either equal to or very close to the same size as normal human ADA as shown by their comigration with authentic ADA (Fig. 3). Our sizing gel could resolve differences in molecular weight of 1,000 or less. This is equivalent to ~10 amino acids in the protein or 30 base pairs in the structural gene. The ADA from the normal B lines, GM-130, GM-131, and GM-2184, was more difficult to visualize than ADA from other cell lines because of the relatively low amount of ADA-specific mRNA in these lines (Table II).

Comparisons of the ADA catalytic activity in extracts of cells grown in culture with the amount of ADA protein in extracts by radioimmunoassay and amounts of mRNA by in vitro translation are shown in columns 5, 6, and 7, Table II. The catalytic activity found in GM-3043 cells was higher than previously found in our laboratory (7) and by others (23). The activity of ADA that is detected in this cell line varies considerably from preparation to preparation of cells in bulk, probably because of thermolability of the mutant protein (23). This property was maintained by the GM-3043 cells in culture in our laboratory. Heating at 54°C for 20 min resulted in loss of 96% of the ADA catalytic activity in extracts from GM-3043 cells to be

compared with 48% loss of activity in extracts from GM-2184 cells.

DISCUSSION

ADA activity is deficient in the most common form of hereditary severe combined immunodeficiency disease (1–3). However, the molecular basis of ADA deficiency is not well understood. Evidence of mutations in the structural gene for ADA has been gained by determining the ratios of ADA protein to catalytic activity in cells and cell lines from people with ADA deficiency (7–10) and by demonstrating ADA proteins with decreased heat stability in cells from some patients with hereditary ADA deficiency (23). Recent studies from this laboratory have provided evidence for at least three different mutations in the human ADA gene as causes of ADA deficiency (7). Unfortunately the amount of ADA protein in mutant cells has not been sufficient to permit analyses of peptide maps and amino acid sequences. In most cases of deficient ADA catalytic activity, it is not certain whether the primary defect is in the structure of ADA, its rate of synthesis, or its rate of degradation. To investigate the synthesis of normal and mutant forms of ADA, the present study of the ADA mRNAs and their translation products was initiated. The goals of this study were to determine if mRNA for human ADA could be prepared from human lymphoblasts and translated in vitro; to determine and compare the molecular size and amounts of the ADA proteins synthesized by in vitro translation of the mRNAs from normal and ADA-deficient cell lines; and to compare the amounts of ADA-specific mRNA, ADA protein, and ADA activity in normal and ADA-deficient cell lines.

Seven lymphoblast cell lines were examined. Two

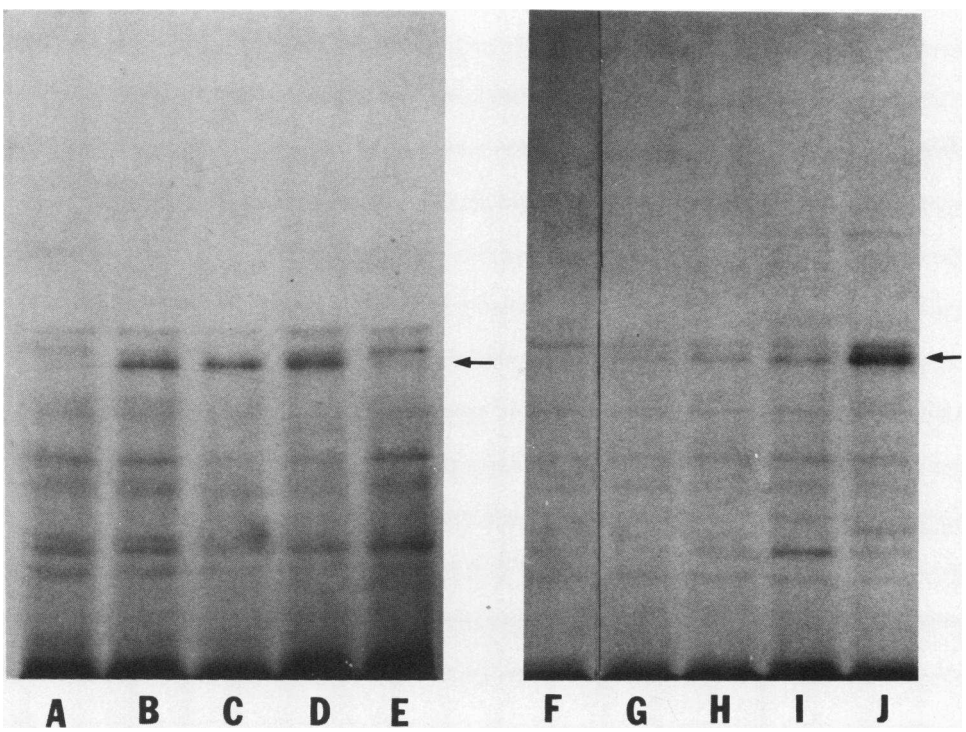

FIGURE 3 Demonstration of ADA as an in vitro product of translation of poly(A)$^+$RNA from normal and ADA-deficient cell lines. In vitro translation reactions were for 50 min. All RNA concentrations were 20 μg/ml, and reaction volumes were 100 μl for lanes A through E and 300 μl for lanes F through J. Translation products were immunoprecipitated, electrophoresed on polyacrylamide-SDS gels, and visualized by autoradiography. The arrows mark the position of purified human ADA. Lanes A and F were derived from in vitro translation products treated with nonimmune rabbit serum (4 μl). All other lanes were derived from in vitro translation products treated with antiserum against native human ADA (4 μl). Lanes A and B, poly(A)$^+$RNA from CCRF-CEM cells. Lane C, poly(A)$^+$RNA from HPB-ALL cells. Lane D, poly(A)$^+$RNA from GM-3043 cells. Lane E, poly(A)$^+$RNA from GM-2184 cells. Lanes F and J, poly(A)$^+$RNA from GM-2606 cells. Lanes G, H, and I, poly(A)$^+$RNA from normal B cell lines, GM-130, GM-131, and GM-2184, respectively.

lines, CCRF-CEM (24) and HPB-ALL (25), had the highest levels of normal ADA protein and catalytic activity. They were originally derived from children with acute lymphoblastic leukemia. Epstein-Barr virus-transformed B lymphocytes from healthy individuals were the sources of the three normal B lines. GM-130, GM-131, and GM-2184 were obtained from a 25-yr-old male, a 23-yr-old female, and a 36-yr-old male, respectively (26). These normal B lines have intermediate amounts of ADA catalytic activity and immunoreactive protein. Two ADA-deficient, transformed B lymphoid cell lines were examined. The GM-3043 cells were derived from lymphocytes of a 16-year-old !Kung tribesman from South Africa (26). The patient had very low levels of ADA activity in his erythrocytes, but normal immunological function. His lymphocytes contained 20–30% of normal ADA activity (27). The second ADA-deficient cell line, GM-2606, was derived from lymphocytes of an 8-mo-old immunodeficient boy (26). These cells contain very low levels of enzyme activity and protein.

RNA was isolated from the seven different lymphoblast cell lines and poly(A)$^+$RNA was prepared from the total RNA of each line. Autoradiographs of polyacrylamide-SDS gels of electrophoresed, immunoprecipitated in vitro translation products from the poly(A)$^+$RNA preparations of all seven cell lines revealed that ADA had been synthesized. Therefore, all of the poly(A)$^+$RNA preparations contained ADA mRNA. Verification of the synthesis of ADA was obtained through demonstration of (*a*) specific immunoprecipitation of newly synthesized protein using three different antisera, one rabbit antiserum directed against native human ADA, one rabbit antiserum directed against SDS-denatured ADA, and one goat antiserum against native human ADA; (*b*) comigration of bands of newly synthesized protein with authentic ADA when immunoprecipitated translation products were electrophoresed on SDS-gels; and (*c*) competition between the newly synthesized radioactive ADA and purified homogeneous nonradioactive human ADA for antibody binding sites. Competition between purified authentic ADA and newly synthesized ADA was demonstrated for translation products of mRNAs from four different cell lines (Fig. 2 and HPB-ALL, data not shown). Competition experiments were not performed with the other three lines.

Although the size of mature ADA protein has been reported previously for normal lymphoid cells and for GM-3043 cells (7), this study is the first report of the size of the mutant ADA protein from GM-2606 cells. The in vitro synthesized ADA proteins were all of the same or very close (i.e., ±1,000 mol wt) to the same molecular size as purified ADA from human leukocytes because they comigrated with this protein on denaturing gels. Because the ADA translation product was of normal size when synthesized in reticulocyte lysates that do not carry out posttranslational modifications, there is normally little or no posttranslational processing of ADA protein in lymphoid cells. The mutations that cause ADA deficiency in the cells from the !Kung child, GM-3043, and the cells from the immunodeficient child, GM-2606, are not defects that cause detectable size changes in the protein product. Deletions or insertions of bases into DNA would have to be smaller than 30 base pairs. The mutations both could be point mutations in the structural gene resulting in the change of a single amino acid in the protein. In the case of the GM-2606 cells, such an amino acid change in the protein may be at or near the active site of the enzyme because extracts of these cells have up to one-tenth the normal amount of ADA protein, but less than one-hundredth the normal ADA catalytic activity. In the case of the GM-3043 cells a substituted amino acid is likely to be at a site other than the active site because extracts of these cells sometimes have normal or even above normal activity, although the activity is abnormally thermolabile (reference 23 and Results). Such an amino acid substitution could induce a conformational change in the molecule that would lead to more rapid denaturation and degradation. The amount of radioactivity incorporated into the translation products is inadequate to permit direct structural analysis by autoradiographs of peptide maps.

The relative amounts of a specific protein synthesized in vitro have frequently been used to estimate relative amounts of specific mRNAs, or more accurately, specific translatable mRNAs in poly(A)$^+$RNA preparations (15, 28–30). The relative amounts of translatable mRNAs for ADA (column 4, Table II) were found to parallel closely the relative ADA catalytic activities and ADA protein content of cells in the normal B cell lines and the T lymphoblast lines. This close correlation between intracellular enzyme levels and translatable mRNA levels in the normal B cell line and T lymphoblast lines could most readily be explained if the in vivo levels of ADA were regulated almost totally by the amount of specific mRNA present, and consequently by the rate of ADA synthesis. This explanation would contrast with the findings of Daddona (31) who reported a twofold greater rate of synthesis and a three- to sixfold slower rate of degradation of ADA in T lymphoblast lines as compared with B lymphoblast lines. His estimates of rates of synthesis were based on in vivo pulse-labeling techniques, whereas ours are based on relative mRNA content. Data presently available are not adequate to establish with certainty the relative contributions of synthesis and degradation to regulation of amount of ADA pres-

ent in lymphoblasts. All available data, however, do assign an important role to synthesis.

The relative amounts of translatable ADA mRNA in the two mutant, ADA-deficient cell lines were three to fourfold greater than in the normal B cell lines and half that of the T lymphoblast lines (Table II, column 5). When compared with in vivo steady-state levels of the mutant ADA proteins, the relative amounts of ADA protein synthesized by in vitro translation were from three- to 33-fold more than the greatest relative amounts of ADA protein that we have detected in cell extracts by radioimmunoassay (Table II columns 5 and 7). However, in the caes of the cell derived from the !Kung child, the GM-3043 line, an occasional catalytic and radioimmunoassay has revealed more ADA activity and protein than found in the normal B cell lines. That the level of translatable ADA mRNA would be higher than the relative amount of ADA protein in GM-3043 cells was not entirely unexpected because of the evidence that the cells could make a thermolabile ADA in substantial quantities. That the ADA specific mRNA in GM-3043 would be three-fold higher than ADA specific mRNA in normal B lymphoblasts was unexpected. The finding of levels of translatable ADA mRNA in the GM-2606 cells that were three-fold greater than in the normal B cell lines was entirely unexpected because the most ADA protein that had been detected in extracts of these cells was only one-tenth that of the normal B cell lines (reference 7 and Table II). The relative amounts of translatable ADA mRNA and actual percentages of ADA synthesized in vitro in the two mutant cell lines indicate that the deficient levels of ADA protein found in these cells in vivo are not caused by deficient mRNA levels; in fact, three-fold greater than normal levels of ADA mRNA appear to be present. The deficient levels of ADA protein in cell extracts in the presence of increased mRNA and increased rate of synthesis could be explained if defective mutant proteins are made that are abnormally unstable and rapidly degraded. The finding of an increased rate of enzyme synthesis associated with a structural mutation would not be unprecedented. In 1970, Yoshida (32) reported that a single amino acid substitution, histidine to tyrosine, in human glucose-6-phosphate dehydrogenase was associated with a fourfold increase in concentration and rate of synthesis of the protein in cells. Whether the increased synthesis was secondary to increased amounts of mRNA in cells or to an increased efficiency of translation was not determined. Apparently the mutant glucose-6-phosphate dehydrogenase was as stable as the normal enzyme in contrast to the two ADA mutant proteins we have studied. Melton et al. (33) demonstrated a 25- to 50-fold overproduction of hypoxanthine/guanine phosphoribosyl-transferase in in vitro translations of poly(A)$^+$ RNA from a mouse neuroblastoma revertant cell line. This overproduced enzyme was also structurally defective as indicated by greatly reduced thermal stability relative to the wild type mouse enzyme.

The variation in translatable ADA mRNA in our seven lymphoblast cell lines may be caused by actual differences in mRNA levels or by different efficiencies of translation of similar amounts of mRNA. However, studies of CAD enzyme (a multifunctional protein containing carbamyl-P-synthetase, aspartate transcarbamylase, and dihydroorotase activities) (34) and dihydrofolate reductase (35) have shown a close correlation between levels of enzyme protein and specific mRNA in a variety of cell types. Whether this correlation exists for ADA in normal and mutant cell lines remains to be shown. If the amounts of adenosine deaminase mRNA are actually increased in the mutant B lines and leukemic T lines over the normal B lines, the question of the mechanisms of this increase in specific messenger arises. It could be caused by more efficient transcription of the DNA, by differences in processing and degradation of the mRNA, or perhaps even by amplification of the ADA gene in cells with unusually high ADA activities.

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