Cell type-specific transcriptional regulation of the human adenosine deaminase gene

David L.Lattier, J.Christopher States, John J.Hutton and Dan A.Wiginton*

Children's Hospital Research Foundation and Department of Pediatrics, University of Cincinnati
College of Medicine, Cincinnati, OH 45229, USA

Received October 12, 1988; Revised and Accepted January 3, 1989

ABSTRACT

The relative rates of transcription of the human adenosine deaminase (ADA) gene were determined in isolated nuclei from T and B lymphoblasts and skin fibroblasts. ADA gene transcription occurs at higher rates in T cells than in B cells and fibroblasts. Relative steady state ADA mRNA levels were also determined for each cell line, and these values were found to correlate with relative rates of transcription of the gene. Therefore, the primary mechanism for control of expression of this ubiquitous enzyme is at the level of transcription. The ratios of ADA enzymatic activity to specific mRNA content were also compared between cell lines. The B lymphoblasts exhibited lower ratios than did the T lymphoblasts, suggesting that rates of protein degradation were several fold greater in B than in T lymphoblast cell lines. This finding is consistent with previous direct measurements of ADA protein turnover. Differential rates of protein turnover in B as compared to T cells provide a secondary mechanism for the regulation of ADA expression. In addition to transcription initiation being the major control mechanism of steady state ADA mRNA in all cell lines, first intron elongation pausing occurs in fibroblasts, and discrete regions of RNA polymerase II and RNA polymerase III antisense transcripts are observed in all cell lines studied.

INTRODUCTION

Adenosine deaminase (ADA, adenosine aminohydrolase, EC 3.5.4.4) is a catabolic enzyme in the purine salvage pathway which catalyzes the irreversible deamination of adenosine and deoxyadenosine. Profound deficiency of ADA activity in humans causes an autosomal recessive form of severe combined immunodeficiency disease (SCID). The clinical and metabolic characteristics of hereditary ADA deficiency have been thoroughly studied (1,2). Our group recently cloned and sequenced the entire normal human ADA gene (3). It contains 12 exons, dispersed within 32 kb of DNA. The mRNA encoded by this gene is approximately 1500 nucleotides, within which is a translatable sequence of 1089 nucleotides that encodes a protein of 40,762 daltons (4).

Our interest in defining the regulatory mechanism of the ADA gene stemmed from several initial observations. Immunofluorescence and immunoperoxidase assays indicated that all T cell leukemia lines tested were strongly stained for the enzyme, whereas weak staining was seen in Epstein Barr virus transformed B cell lymphoma lines as well as non-T, non-B cell leukemia lines (5). This indicates that the content of ADA protein varies many fold among various types of cells. Levels of translatable ADA mRNA also vary among various B and T cell

lines (6,7). T lymphoblast translatable poly(A)+ ADA mRNA was estimated to be 0.01% of total mRNA. In contrast, B cell lymphoblast translatable poly(A)+ ADA mRNA was estimated to be five to seven-fold lower. The relative amounts of translatable mRNAs for ADA were reflected in the relative ADA catalytic activities and immunoreactive protein levels of B and T lymphoblasts (6). The correlation between intracellular enzyme and translatable mRNA levels in B and T lymphoblast lines could result if *in vivo* levels of ADA protein were regulated largely by specific mRNA content.

Previous ADA mRNA quantitations obtained by comparing relative amounts of immunoprecipitable *in vitro* translation product were unable to address the possibility of differential mRNA turnover rates between T and B lymphoblasts. The present study is an attempt to understand the underlying bases for the relative levels of steady state ADA mRNA in lymphoblast cell lines. The ADA mRNA level could be a function of transcriptional rate alone (8,9), or could involve posttranscriptional mechanisms that direct further processing and differential stability of mRNA. In this report, we demonstrate that the major control mechanism of ADA expression in the cell lines tested is at the level of transcription.

MATERIALS AND METHODS

Cell culture.

GM2219C (Molt-4) and GM3671B (CCRF-CEM), both of which are T marked human acute lymphoblastic leukemias, GM3638A (Epstein Barr virus transformed human B cells), GM4671 (Raji, Burkitt lymphoma), and GM4429B (SV40 transformed human skin fibroblast), were obtained from the Human Genetic Mutant Cell Repository, Camden, NJ. Leukemic lymphoblasts were maintained in suspension culture at 37°C, 5% CO₂ in RPMI 1640 (Flow Laboratories) without antibiotics, supplemented with 10% fetal bovine serum (Hyclone) and 2 mM L-glutamine. Cultures were grown to a density of 5-6 x 10⁵ cells/ml before harvest. Fibroblasts were grown to confluence in α -modification minimal essential medium (Flow Laboratories) with 10% fetal bovine serum (Hyclone). They were harvested by trypsinization, and trypsin was inactivated by washing the cells with media containing 10% serum.

Isolation of nuclei.

Nuclei were isolated at 4°C as described by Marzluff et al. (10). Briefly, the procedure was as follows: Suspensions of cells were pelleted by centrifugation at 50 x g for 20 min and washed three times in 30 mM Tris-HCl pH 7.4, 120 mM KCl, 5 mM Mg-acetate, and 7 mM 2-mercaptoethanol. Pellets containing approximately 5 x 10⁸ cells were resuspended in 3 ml of 0.3 M sucrose containing 2 mM Mg-acetate, 3 mM CaCl₂, 10 mM Tris-HCl pH 7.5, 0.1% triton X-100 and 0.5 mM 2-mercaptoethanol. The cells were disrupted with 10 strokes of a Dounce homogenizer using a B type pestle. To the homogenate was added an equal volume of 2 M sucrose containing 5 mM Mg-acetate, 10 mM Tris-HCl pH 7.5, and 0.5 mM 2-mercaptoethanol. This mixture was layered over 1.5 ml of the 2 M sucrose buffer and centrifuged for 55 min. at

65,000 x g, at 4°C in a Beckman SW60 rotor. The pelleted nuclei were washed and resuspended at a concentration of 2×10^8 /ml in 25% glycerol containing 5 mM Mg-acetate, 0.1 mM EDTA, 5 mM 2-mercaptoethanol, and 50 mM HEPES/NaOH pH 7.5. Aliquots (0.1 ml) of resuspended nuclei were quick frozen in liquid nitrogen and stored at -70°C in 1.5 ml Eppendorf tubes.

In vitro chain elongation.

Nuclei, 2×10^7 for autoradiographic analyses and 4×10^7 for liquid scintillation quantitation, were incubated in 0.5 and 1.0 ml reaction mixtures, respectively, having the following composition essentially as described by Mory and Gefter (11): 7.5% (w/v) glycerol, 19 mM HEPES/NaOH (pH 7.5), 3.8 mM Mg-acetate, 84 mM KCl, 30 μ M EDTA, 3 mM 2-mercaptoethanol, 10 mM NH_4Cl , 130 μ g/ml creatine phosphokinase, 10 mM creatine phosphate, 1 mM ATP, 250 μ M CTP and UTP, 0.5 mM S-adenosyl-L-methionine and 0.33 μ M [α - ^{32}P]GTP (~3,000 Ci/mmol; New England Nuclear, Boston, MA.) Reaction mixtures were incubated at 25°C for appropriate times. Labeling reactions were terminated by addition of RNase-free DNase I (RQ1, Promega Biotech, Madison, WI.) to a final concentration of 144 U/ml. Following incubations, nuclei were lysed under the conditions described to us by Michael Briskin, University of California, Los Angeles: 42% (w/v) urea, 350 mM NaCl, 10 mM Na-acetate (pH 5.2), 1 mM EDTA, 1% sarkosyl, and 5% equilibrated phenol. After extraction with phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol) and ethanol precipitation, ^{32}P labeled hnRNA was again subjected to RNase-free DNase I treatment for an additional 30 min. Following a series of phenol-chloroform-isoamyl alcohol (25:24:1, vol/vol/vol), and chloroform-isoamyl alcohol (24:1) extractions, RNA was isolated by 2-3 2.5 M ammonium acetate/ethanol (1:2.5) precipitations.

Transcription probes.

The single stranded DNA probes derived from cloned human ADA gene fragments are shown in Fig. 1A. Probe I is a 1.1 kb *Pst*I fragment (bases 31,262 to 32,393 of the ADA gene) (3) subcloned into bacteriophage M13mp18. Probe II is a 2.0 kb *Bgl*III-*Hind*III fragment (bases 30,299 to 32,297 of the ADA gene) subcloned into M13mp18. Base numbers of probe regions refer to distance from the major transcription initiation site. Probe I spans the region from the 3' end of exon 11 to a point 353 bp 3' of the poly A site. Probe II contains exons 10, 11 and 12. Probe I strand sense was determined by sequence analysis, and probe II strand sense was determined by testing for complementarity with probe I. The entire region defined by probes I and II contains no repetitive elements. The actin probe used in autoradiographic analyses is a 365 bp fragment from the 3' untranslated region of γ -cytoplasmic actin (provided by Dr. Larry Kedes, Stanford University, Stanford, CA). ADA genomic probes 1-7 (Fig. 5 - hatched regions, nos. 1-7) used in the autoradiographic 'gene scan' were also subcloned into M13. Similar to the autoradiographic transcription analysis, each probe was cloned in both sense and antisense orientations relative to the ADA primary transcript.

Preparation of membranes.

For determination of transcription rates by autoradiography and scintillation spectrometry, single stranded DNAs described above were applied to S&S BA85 nitrocellulose membranes by the procedure described in Schleicher and Schuell Application Update No. 372. DNAs were applied by hand using 100 μ l capillary pipettes, and each "dot" application contained 4 μ g total ssDNA. For the 'gene scan' detection of antisense transcripts in the ADA gene, 5 μ g total ssDNA of probes 1-7 (Fig. 5, hatched regions), were applied to S&S 0.45 μ m Nytran membrane using an S&S Minifold II apparatus per recommendation of the manufacturer.

Synthesis of ^3H labeled cRNA.

The probe II fragment (Fig. 1A) was cloned into the Genescribe pT7-2 vector (United States Biochemicals, Cleveland, OH) in ADA mRNA sense orientation. ^3H -cRNA was prepared by transcription of *Hind*III linearized plasmid by T7 RNA polymerase (Pharmacia, Piscataway, NJ) according to instructions in Pharmacia Molecular Biology Division catalog No. 27-0801. The transcription reaction contained 400 μ M each of GTP, ATP, CTP and UTP, including 120 μ Ci (64 μ M) of [5,6 ^3H]UTP (36.5 Ci/mmol; New England Nuclear, Boston, MA).

Hybridization conditions.

Hybridization of membrane-bound ssDNA probes to labeled nuclear hnRNA products was carried out as follows. ^{32}P -labeled nuclear RNAs extracted from 4×10^7 nuclei were dissolved in 330 μ l of hybridization buffer containing 50% formamide, 5X SSPE (1X SSPE = 0.150 M NaCl, 0.010 M NaH_2PO_4 , 0.001 M EDTA [pH 7.4]), 0.1% polyvinylpyrrolidone 10, 0.1% Ficoll 400, 0.5 mg/ml yeast tRNA, and 0.5% SDS. This mixture was then overlaid on nitrocellulose membranes which had been pre-hybridized in a separate aliquot of the above buffer for a period of 24 h. Hybridizations in the multiple probe 'gene scan' experiments were essentially as described, with the exception of increased hybridization volume necessary to accommodate a greater membrane surface area. In direct quantitation experiments, 1.05×10^5 cpm of [5,6 ^3H]UTP cRNA (prepared as described above) was added to the hybridization buffer containing ^{32}P labeled nuclear RNA in order to monitor hybridization efficiency in each experiment. Hybridizations were incubated at 42°C for a period of 24 h. Following hybridization of labeled RNA to membrane bound probes, membranes were washed twice at room temperature for 10 min, in PES buffer (5 mM Na-phosphate (pH 6.4), 1 mM EDTA and 0.1% SDS). Membranes were then washed twice in 1X SSC (1X SSC = 0.150 M NaCl, 0.015 M Na-citrate [pH 7.0]) containing 5 U/ml T1 RNase plus 5 μ g/ml RNase A (both from Boehringer-Mannheim) for 1 hour at 42°C. All nitrocellulose membranes were washed in a final 200 ml of PES buffer for a period of 1 hour at 50°C. Membranes to be autoradiographed were hybridized for 72 h, and afterwards blotted on Whatman 3MM paper and exposed to Kodak XAR film (Eastman Kodak Co., Rochester, N.Y.) with two Lightning Plus intensifying screens for a period of 24 h at -70°C (12). Membranes used for determination of relative transcription rates in parts per million (ppm) by ^3H & ^{32}P scintillation counting were prepared in the following

manner: Hybridized "dots" were cut from membranes with a small gauge cork borer and each was solubilized in 160 μ l 2-methoxyethanol for a period of 30 min. To each solubilized nitrocellulose "dot" was added 4.5 ml Aquasol-2 (New England Nuclear LCS Application Note No. 48, Boston, MA.) and samples were counted in a Beckman LS-7000 scintillation counter. Calculation of the relative rates of transcription in parts per million was accomplished using the following equation (13):

$$\frac{\text{SPECIFIC ADA HYBRIDIZATION}}{\text{TOTAL cpm PRESENT IN HYBRIDIZATION MIX}} \times \frac{\text{LENGTH of ADA PRIMARY TRANSCRIPT}}{\text{LENGTH of ADA SPECIFIC PROBE}} \times 10^6$$

In this equation, specific ADA hybridization = 32 P cpm bound to ADA specific probe minus average 32 P cpm bound to mp18 DNA, corrected to 100% efficiency of hybridization; total cpm present = acid precipitable cpm 32 P chain elongation products available for hybridization in hybridization reaction mix; length of primary transcript = 32 kb from the major transcription initiation site to the poly A site (3); length of probe = 2 kb (Fig. 1A).

Membrane transfer and analysis of genomic DNA.

*Hind*III digested genomic DNA from each cell line was electrophoresed in 0.8% agarose and blotted onto S&S 0.45 μ m Nytran in 25 mM Na-phosphate (pH 6.7). Remaining manipulations of blotted membrane were essentially as described by manufacturer. The membrane was hybridized separately with random primer radiolabeled ADA cDNA (Fig. 1B) and a 365 bp 3' untranslated region of γ -cytoplasmic actin (provided by Dr. Larry Kedes, Stanford University, Stanford, CA) in hybridization conditions described above. Following hybridization to ADA cDNA and autoradiography, the membrane was stripped of probe by incubation in PES buffer, two times for 10 min. at 90°C. The membrane was then hybridized a second time with γ -cytoplasmic actin probe. After each hybridization, the membrane was washed twice for 15 min. at 42°C in PES and autoradiography was performed as described above, but for only 17 h. The autoradiograms of both hybridizations (Fig. 3) were overlayed for comparison and analyzed with a Hoefer GS 300 scanning densitometer, interfaced with Hoefer GS 370 Data System and an Apple Macintosh computer.

Determination of relative cytoplasmic ADA mRNA levels.

Detection of ADA specific cytoplasmic RNA was carried out essentially as described in Schleicher and Schuell Application Update number 372. Aliquots of diluted cytoplasmic extracts were applied to a nitrocellulose membrane using an S&S Minifold II apparatus and hybridized to a 1.4 kb *Nco*I restriction fragment of cloned ADA cDNA (Fig. 1B) (14) radiolabeled by the random primer method (15). The blots were autoradiographed by exposure for 24 h. Autoradiograms were scanned with a Hoefer light densitometer and areas under peaks were obtained using a Hewlett Packard integrator.

Determination of ADA enzymatic activity.

Whole cell extracts were assayed for ADA enzymatic activity as described previously (16). [^{14}C] adenosine substrate and [^{14}C] inosine products were separated by descending chromatography on Whatman DE-81 paper in 1 mM ammonium formate running buffer. Specific activity of ADA is reported as nmol of adenosine converted to inosine per min per 10^8 cells.

RESULTS AND DISCUSSION

The time course of incorporation of radiolabeled nucleotides into RNA by isolated nuclei was determined for each cell line. *In vitro* chain elongation reactions were performed and aliquots of each were taken at ten minute intervals. Maximal incorporation of radiolabel into TCA precipitable product occurred at 20 min. in all lymphoblast nuclei and at 40 min. in fibroblast nuclei. The times of maximal incorporation were used as incubation times in nuclear chain elongation reactions for determination of relative transcription rates of the ADA gene in nuclei from the various cell lines. The nuclei from all cell lines incorporated a maximum of 5-7% of added radiolabeled GTP. The inclusion of 50 ng/ml α -amanitin, a potent inhibitor of RNA polymerase II, reduced maximum ^{32}P incorporation by 69-71% in nuclei from all cell lines, and totally inhibited ADA specific transcription. In order to establish an estimate of the relative transcription rates in the various cell lines, ADA RNA synthesized in nuclei *in vitro* was detected by autoradiographic means. Nuclei (2×10^7) from each cell line were incubated with 0.5 mCi [α - ^{32}P]GTP as described in Material and Methods to produce ^{32}P -labeled primary transcripts. Primary transcripts were hybridized to cloned sense and antisense single stranded ADA genomic probes corresponding to probe I (Fig. 1A). As internal controls, the ^{32}P primary transcripts were also hybridized to human γ -cytoplasmic actin sense and antisense probes. The antisense strand ssDNA probes detect primary transcripts of the respective genes. Molt 4, GM3638 and GM4429 were compared in one experiment (Fig. 2A), and CCRF-CEM and Raji in another (Fig. 2B). Four identical nitrocellulose membranes, to which ADA and cytoplasmic actin probes were bound, were prepared and hybridized to fractional portions of *in vitro* synthesized RNA from each cell line. These fractions contained increasing amounts of ^{32}P labeled primary transcripts (see legend for Fig. 2). This method, which utilizes an equivalent amount of total cpm per hybridized membrane, permits visual determination of relative fold differences in transcription among cell lines. The intensities of the autoradiographic signals from these hybridization experiments yielded an estimate of the relative sense and antisense transcription rates of the ADA and γ -cytoplasmic actin genes in these cells. Signal from hybridization detecting antisense transcripts from the ADA gene is readily apparent in all cell lines. Antisense transcripts of the actin gene are visible in CCRF-CEM cells, and only faintly in other cell lines on those membranes hybridized with greater concentration of radiolabeled elongation products. In all cases, the intensities of the actin gene antisense transcripts are lower than the ADA counterparts. Therefore, hybridization to the actin ssDNA sense probe should be used as a visual

FIG. 1. Probes used in analysis of expression of the human ADA gene. (A) 3' end of the ADA gene (3). Exons 6 through 12 and regions of intervening sequence are depicted. Region I in brackets denotes a 1.1 kb *Pst*I fragment in M13 (sense and antisense) used for autoradiographic analysis of rates of transcription. Region II in brackets denotes a 2.0 kb *Bgl*III - *Hind*III fragment in M13 used in quantitative analyses of transcription rates. Nucleotide sequence of the ADA gene is known through the end of the light line below the exonic regions. The arrow represents the last Alu repetitive element in the ADA gene. O, *Xho*I; G, *Bgl*III; H, *Hind*III; X, *Xba*I. The 35 kbp mark refers to the distance from the beginning of sequence analysis (3). Sequence analysis began approximately 4 kb 5' of the major transcription initiation site. (B) Probe used in detection of cytoplasmic mRNA is the 1.4 kb *Nco*I fragment of ADA cDNA (14). Depicted is the full length cDNA with proportional exonic regions. N, *Nco*I; B, *Bam*HI; P, *Pst*I.

reference to non-specific background hybridization. Antisense transcription in the ADA gene will be discussed in detail later in the text.

There were higher levels of *in vitro* ADA transcription in human T lymphoblast (Molt 4 & CCRF-CEM) nuclei than B lymphoblast (GM3638A & Raji) nuclei and fibroblast (GM4429B) nuclei (see Fig. 2). Visual inspection of Fig. 2A indicates that the intensity of ADA signal from the T line Molt 4 1X reaction is comparable to the 8X signals for both the B line GM3638A and the fibroblast line GM4429B. This indicates an approximate 8-fold difference in transcription rates. The 1X and 2X ADA signals of the T line CCRF-CEM is visually equivalent to the 4X and 8X signals of Raji B line (Fig. 2B). This indicates an approximate 4-fold difference in transcription rates.

FIG. 2. Autoradiographic analysis of transcription of the human ADA gene in five cell lines: Shown are four 2 cm² membranes per cell line. A single "dot" of each probe, as indicated at the bottom right of the figure, was applied to each membrane. Each membrane was hybridized with a fractional portion of the total elongation product from a single incubation (1X=1/15, 2X=2/15, 4X=4/15, 8X=8/15). (A) Molt 4 (T cell), GM3638 (B cell), and GM4429 (fibroblast). 1 X=2.5 x 10⁶ cpm/ml. (B) Raji (B cell) and CCRF-CEM (T cell). 1 X=5.5 x 10⁶ cpm/ml.

It should be noted that 3' regions of the ADA gene were chosen as probes in order to determine relative production of primary transcripts in each cell line. In the absence of transcriptional control mechanisms, such as intragenic polymerase pausing, the rate limiting step of transcription is presumably initiation by RNA polymerase II. Even in the event of mechanisms affecting transcription elongation rates of the ADA gene, 3' genomic probes detect the production of completed primary transcripts.

Of the cell lines used to determine relative rates of transcription, only the Raji cells are diploid. It was necessary to insure that greater transcriptional activity observed in the T cell lines did not result from selective amplification of the ADA gene. High molecular weight DNA isolated from 2 x 10⁶ nuclei obtained from each cell line was digested with *Hind*III. Following agarose gel electrophoresis and transfer to Nytran membrane, the membrane was probed separately with ADA cDNA (Fig. 1B) and γ -cytoplasmic actin probes. The respective autoradiograms were overlaid (Fig. 3), and scanning light densitometry was used to determine ratios, in arbitrary units, of total ADA to γ -cytoplasmic actin in all cell lines. The minimum and maximum values in the respective cell lines varied by approximately 1.5, indicating that the

FIG. 3. *Hind*III genomic digests of DNA isolated from 2×10^6 nuclei per cell line. Membrane was probed separately with ADA cDNA and γ -cytoplasmic actin at 1×10^6 dpm/ml. Lanes: M, *Hind*III and *Bst*E2 cut lambda phage DNA; A, T cell (CCRF-CEM); B, T cell (Molt 4); C, B cell (GM3638); D, B cell (Raji); E, Fibroblast (GM4429). Autoradiograms from independent hybridizations (described above) were overlayed for the purpose of comparison.

relative copy number of ADA and γ -cytoplasmic actin is not a function of gene amplification in those lines analyzed. The identical actin fragment used as ssDNA probe in autoradiography of elongation products (Fig. 2 A and B) shows that γ -cytoplasmic actin is transcribed at similar rates in lymphoblasts, and higher in fibroblasts.

In order to quantify ADA transcriptional rates in the cell lines, *in vitro* chain elongation reactions were altered in the following manner to permit scintillation counting of labelled RNA products. Probe II (Fig. 1A) was used to detect hybridizable ADA primary transcripts. This permitted the hybridization of a greater number of ^{32}P counts than did Probe I because of the increase in length of Probe II. Also, the number of nuclei and the amount of isotope were doubled. Higher RNA concentrations present in the hybridization mix, resulting from an increase in the number of nuclei, raised the efficiency of ADA specific hybridization. $[5,6^3\text{H}]\text{UTP}$ cRNA, an inverse complement to the filter bound ADA probe, was used to assess hybridization efficiency in each chain elongation reaction. Hybridization efficiency was routinely 5-8% of input radiolabeled cRNA. Previous groups have reported hybridization efficiencies of cRNA transcripts in the range of 45-50%. These control transcripts are typically generated by *E. coli* RNA polymerase on denatured plasmid templates which contain a portion

TABLE 1. Rate of ADA gene transcription, level of ADA mRNA, and catalytic activity of ADA in cells.

Cell line (type)	Input in cpm, a (x10 ⁻⁸)	Cpm hybridized	Relative transcriptional rate of the ADA gene in ppm ^b	Level of ADA mRNA ^c	Catalytic activity of ADA ^d	Steady state mRNA transcriptional rate	Steady state ADA activity/ steady state mRNA
Molt 4 (T cell)	1.58	157 ± 5	198	250	9730	1.3	39
CCRF-CEM (T cell)	2.40	37 ± 6	49	116	1800	2.4	16
GM3638 (B cell)	1.88	11 ± 3e	<20e	33	217	>2 ^f	7
Raji (B cell)	1.46	9 ± 4e	<20e	36	162	>2 ^f	5
GM4429 (fibroblast)	1.29	7 ± 2e	<20e	16	225	>1 ^f	14

^aTotal in vitro synthesized ³²P nuclear RNA available for hybridization.

^bSee Materials and Methods

^cRNA levels are expressed as integrated peak areas for extracts from 1.25 x 10⁵ cells in arbitrary units.

^dADA catalytic activities are expressed as nmol/min/10⁸ cells. Catalytic activity of ADA has been shown to be directly correlated with level of ADA protein in cells (7).

^ecpm hybridized insignificantly above background; therefore ppm value less than 20.

^fCalculation based on 20 ppm.

of the specific gene, or the cDNA (17). This methodology results in random transcription starts by *E. coli* RNA polymerase, in addition to short transcripts. The obvious difference in hybridization efficiencies reflects the base-length dependent kinetics of filter hybridization. We chose a control transcript equal in length to the filter-bound probe, since this more closely approximates hybridization conditions of primary transcripts. Hybridization of control transcripts is relative, and merely serves as an indicator of experimental consistency. Single stranded M13mp18 phage DNA was used as a negative control to determine background levels of non-specific hybridization. Relative rates of transcription, expressed in parts per million of total cellular transcripts, are shown in Table 1.

The purpose of calculating relative ppm values for each cell line was to provide a means of comparison of steady state mRNAs and rates of transcription. ADA gene transcription was easily detected and quantified by scintillation spectrometry in the T cells (Molt 4 & CCRF-CEM). ADA gene transcription by the B lymphoblasts and fibroblasts was not significantly above the level of background counts. Therefore, quantitative measurements in these cell lines are subject to uncertainty and are listed as being less than 20 ppm (Table 1). This value is considered to be within an appropriate range, and consistent with independent estimations as determined by autoradiography (Fig. 2). Quantitation of relative transcription rates of the ADA gene indicate a 4.2 fold difference between the two T cell lines (Table 1). The ADA gene is transcribed at approximately equal rates in the two B lymphoblast lines and the fibroblast line (Table 1 and Fig. 2). T lymphoblasts transcribe the ADA gene at significantly greater rates than do the B lymphoblasts or fibroblasts. Considering the data presented in Table 1 and Figure 2, Molt 4 cells and CCRF-CEM cells transcribe the ADA gene at approximately 10 times and 4 times the rate of B lymphoblasts and fibroblasts, respectively. Autoradiographic analyses of relative ADA transcription rates in all cell lines were performed with numerous non-repetitive ssDNA probes. Results of all experiments were consistent with data presented in Figure 2. Scintillation spectrometry of hybridized radiolabeled elongation product, as described above, was also performed using a 4 kb probe which extended 2 kb from the 5' end of probe II (Fig. 1B). This 4 kb probe was twice the size of probe II and represented the largest region of the ADA gene without repetitive elements. Use of this probe simply resulted in doubling the ^{32}P counts bound when analyzing T cell transcription. Therefore, when these values of cpm hybridized were applied to the equation presented in Material and Methods, identical transcription rates in ppm were obtained. When radiolabeled elongation products from B cell lines were hybridized to the 4 kb probe, ^{32}P counts were still insignificantly above background.

In order to determine relative steady state levels of ADA mRNA in the various cell lines, cytoplasmic mRNA analysis was performed (Fig. 4) as described in Materials and Methods. Previous studies showed that the levels of ADA mRNA were higher in T cells than in B cells (18); therefore, cytoplasmic extracts were diluted over a greater range for T than for B cells and fibroblasts. Because the ADA enzymatic activities in B cells and fibroblasts were equivalent, the

FIG. 4. Cytoplasmic ADA mRNA levels in five cell lines. Cytoplasmic extracts from the indicated number of cells from each cell line were applied to nitrocellulose membranes and hybridized to an ADA cDNA probe (Fig. 1B).

ADA mRNA levels were anticipated to be similar. The autoradiographs shown in Fig. 4 were scanned and the peak areas were determined by integration. Relative ADA mRNA levels are given in Table 1. Amounts of ADA specific mRNA per cell are higher in T cells than in B cells and fibroblasts. Molt 4 cells have 7.2 - fold and CCRF-CEM cells 3.5 - fold more ADA mRNA per cell than the B lymphoblasts (GM3638 or Raji). These data are consistent with previous reports of 5 - to 7 - fold more ADA mRNA in T than in B lymphoblasts (18). The fibroblasts (GM4429) have approximately one-half the ADA mRNA content of B cells.

Values for ADA transcriptional rates, relative ADA mRNA content, and ADA enzymatic activities are also given in Table 1. ADA enzymatic activity has been shown to be directly proportional to ADA protein levels (D. Wiginton, unpublished results) and is used as an indirect measure of ADA protein. The ratios of relative levels of ADA mRNA to rates of ADA transcription and of ADA catalytic activities to relative levels of ADA mRNA are also presented. Transcriptional rates and specific mRNA content vary 10 and 15 - fold, respectively, among cell lines. There is less than a 2.5 - fold difference among cell lines in the ratios of ADA mRNA levels to ADA gene transcription rates. From these calculations, the rate of ADA mRNA degradation is approximately equivalent in all cell lines. The differences among cell lines are probably not significant, but are secondary to the error in measurement of transcription rates. Therefore, steady state ADA mRNA levels in each of the cell lines analyzed appear to be proportional to transcriptional rates.

T lymphoblasts have greater ADA enzymatic activity per cell than do either B lymphoblasts or fibroblasts (Table 1). Molt 4 cells have approximately 48-fold more ADA activity and CCRF-CEM cells have about 9-fold more ADA activity as B lymphoblasts and fibroblasts. Ratios of ADA enzymatic activities to ADA steady state mRNA levels were calculated for all cell lines. From these calculations, ADA net protein turnover rate (synthesis and degradation) can be inferred to be equivalent in CCRF-CEM and fibroblasts. Both cell lines exhibit diminished ratios when compared to Molt 4. Although apparent protein turnover is

similar in fibroblasts and CCRF-CEM, transcription and steady state mRNA levels are drastically different. Transcription rate and RNA level in fibroblasts are approximately 3-4 and 7-fold lower than in CCRF-CEM. This correlates with observed differences in ADA activity between the two lines (8-fold).

Ratios of ADA enzymatic activities to ADA mRNA steady state levels are equivalent in the two B cell lines, GM3638 and Raji. These ratios are much lower than in Molt 4 cells and indicate a large difference in apparent protein turnover in these lines relative to Molt 4. This inference is consistent with direct measurements of ADA protein turnover in lymphoid cell lines (19). Analyses of the apparent rate of ADA synthesis in the cell lines studied demonstrated a 2-fold faster rate of enzyme synthesis in two T cell lines over that in B cell lines. In addition, the two T lymphoblast lines showed approximately a 3- and 6-fold slower rate of ADA degradation relative to that in B lymphoblast lines. Therefore this study showed that B cell lines exhibited an arithmetic 6- and 12-fold greater turnover rate of ADA protein relative to two T cell lines. The present study presumes differences in protein turnover based on ADA enzymatic activity normalized to RNA levels. The differences in ADA activity to mRNA ratios are due to either a decrease in translation efficiency of ADA mRNA, an increase in protein degradation, or a combination of the two mechanisms in B cells relative to T cells.

The experiments of Figure 2 detected antisense transcription at the 3' end of the ADA gene. In order to test for other antisense transcripts in the ADA gene, various non-repetitive M13 probes (Fig. 5, hatched regions 1-7) of similar length were chosen to autoradiographically "scan" the gene by hybridization to radiolabeled elongation products. Molt 4 and GM3638 (Fig. 6A) not only show a marked contrast between hybridization of primary transcripts (ADA sense), but also hybridization to antisense transcripts within probes 1, 4 and 7 (Fig. 5) in both cell lines. The fibroblast line GM4429 (Fig. 6B) likewise shows antisense hybridization to probe 4, but not to probes 1 and 7. Antisense hybridization in the 3' region of the gene has been observed in fibroblasts (see Fig. 2A) using probes I and II (Fig. 1A). This suggests termination of the fibroblast antisense transcription prior to the region of probe 7. Also in fibroblasts, the ADA primary transcript elongation product hybridizes intensely in the region of probe 1 (Fig. 5). This intense signal is eliminated when RNA polymerase II activity is inhibited with 1 μ g/ml α -amanitin. Transcription initiation in fibroblasts may be at higher levels than in B and T cells (Fig. 6A), and the data suggest intragenic pausing may be occurring, similar to that observed in *c-myc* and *c-myb* genes (20,21).

The nature of the antisense transcripts was investigated in Molt 4 nuclei. When RNA Polymerase II activity was inhibited with 1 μ g/ml α -amanitin (Fig. 7), hybridization is seen with probes 1 and 4, but not probe 7, indicating that the latter is of Pol II origin. Inhibition of RNA polymerase III activity with 100 μ g/ml α -amanitin completely eliminated hybridizations in all probe regions, indicating that antisense transcripts in the regions of probes 1 and 4 are of Pol III origin. Based on antisense hybridization to a fragment of ADA cDNA containing exon 2

FIG. 5. Map of the Human ADA gene (Wiginton et al., 1986), showing entire sequenced region and probes used for transcription analyses. Exons are depicted on the heavy black lines, and the arrows below represent Alu type repetitive elements. Restriction sites: O, *Xho*I; G, *Bgl*III; H, *Hind*III; X, *Xba*I; E, *Eco*RI; B, *Bam*HI; P, *Pst*I (partial listing of sites). Hatched boxes, numbered 1 - 7, represent probes of non-repetitive regions cloned into M13 and used for detection of antisense transcripts (Fig. 6 A, B, & C). Probes: 1=628 bp, *Nco*I - *Xho*I; 2=250 bp, *Bgl*III; 3=421 bp, *Hind*III; 4=535 bp, *Bam*HI; 5=556 bp, *Pst*I; 6=389 bp, *Pst*I - *Bam*HI; 7=849 bp, *Pst*I.

(data not shown), the transcript which hybridizes to probe 4 spans the exon, which is 62 bp in length. It is interesting to note that the probe 4 antisense transcript is at a higher absolute level in T than B cells (Fig. 6A); however, relative to hybridizations of primary transcripts (ADA sense) in the respective cell lines, the probe 4 antisense transcript is higher in B cells. Antisense transcripts in the ADA gene may be operative in post-transcriptional regulation. This mechanism is dependent on concentration, length, and probably position of the antisense RNA relative to the primary transcript. By use of microinjection methodology, a complementary RNA sequence of only 52 nucleotides has been shown to effectively inhibit translation of the HSV-TK gene (22). More recently, antisense RNA has been shown to inhibit splicing of pre-mRNA *in vitro* (23). The latter finding may be critical to ADA regulation since the antisense transcript detected by probe 4 crosses the 3' and 5' splice junctions of exon 2.

FIG. 6. Autoradiographic "gene scan" detection of *in vitro* elongation transcripts in the ADA gene using ssDNA probes as described in Fig. 5 legend. In all autoradiograms, M denotes M13mp18 DNA included as a control for non-specific background hybridization. (A): T cell (Molt 4) and B cell (GM3638), each of which was hybridized with 7×10^6 cpm/ml. Autoradiography time was 48 h. (B): Fibroblast (GM4429) hybridized with 7×10^6 cpm/ml. Autoradiography time was 72 h.

The results of this study show that regulation of ADA expression is complex, and composed of both transcriptional and post-transcriptional regulatory processes. The data presented here is in contrast to conclusions presented in a recent publication (24). These authors presented data suggesting that regulation of the ADA gene occurs posttranscriptionally in the nucleus. In the current study we demonstrate that relative rates of transcription of the ADA gene in five cell lines vary over a 10-fold range. Relative quantities of ADA mRNA vary over approximately the same range as rates of transcription; therefore, the concentration of ADA

FIG. 7. Inhibition of T cell RNA polymerase II with α -amanitin. T cell (Molt 4) hybridized with 3.6×10^6 cpm/ml transcription product in the presence of $1 \mu\text{g/ml}$ α -amanitin. Autoradiography time was 72 h.

mRNA in a cell is controlled primarily by rate of gene transcription. ADA enzymatic activity in the same five cell lines varies over a 50-fold range. Of the 50-fold difference in ADA catalytic activity between one T lymphoblast line (Molt 4) and B lymphoblasts, approximately 10-fold is accounted for by differences in rates of ADA gene transcription that direct the levels of mRNA. A secondary mechanism controlling ADA expression within cells is apparent variation in rates of ADA protein turnover.

ACKNOWLEDGMENTS

Supported by National Institutes of Health research grant HD19919. We are grateful to Michael Briskin, Drs. Bruce Aronow, Donal Luse and Linda Walling for insightful discussions and technical advice. We thank Catlan Thorne and George Jacob for technical assistance.

*To whom correspondence should be addressed at Children's Hospital Research Foundation, IDR Room 713, Cincinnati, OH 45229, USA

REFERENCES

1. Giblett, E.R., Anderson, J.E., Cohen, F., Pollara, B., & Meuwissen, H.J. (1972) *Lancet* **2**, 1067-1069.
2. Martin, D.W., & Gelfand, E.W. (1981) *Ann. Rev. Biochem.* **50**, 845-877.
3. Wiginton, D.A., Kaplan, D.J., States, J.C., Akeson, A.L., Perme, C.M., Bilyk, I.J., Vaughn, A.J., Lattier, D.L., & Hutton, J.J. (1986) *Biochemistry* **25**, 8234-8244.
4. Wiginton, D.A., Adrian, G.S., & Hutton, J.J. (1984) *Nucleic Acids Res.* **12**, 2439-2446.
5. Chechik, B.E., Schrader, W.P., & Minowada, J. (1981) *J. Immunol.* **126**, 1003-1007.
6. Adrian, G.S., & Hutton, J.J. (1983) *J. Clin. Invest.* **71**, 1649-1660.
7. Adrian, G.S., Wiginton, D.A., & Hutton, J.J. (1984) *Hum. Genet.* **68**, 169-172.
8. Darnell, J.E., Jr. (1982) *Nature* **297**, 365-371.
9. McKnight, G.S., & Palmiter, R.D. (1979) *J. Biol. Chem.* **254**, 9050-9058.
10. Marzluff, W.F., Jr., Murphy, E.C., Jr., & Huang, R.C.C. (1973) *Biochemistry* **12**, 3440-3446.
11. Mory, Y.Y., & Gefter, M.L. (1977) *Nucleic Acids Res.* **4**, 1739-1757.
12. Swanstrom, R., & Shank, P.R. (1978) *Anal. Biochem.* **86**, 184-192.
13. Berger, F.G., Loose, D., Meisner, H., & Watson, G. (1986) *Biochemistry* **25**, 1170-1175.
14. Adrian, G.S., Wiginton, D.A., & Hutton, J.J. (1984) *Mol. Cell. Biol.* **4**, 1712-1717.
15. Feinberg, A.P., & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6-13.
16. Coleman, M.S., & Hutton, J.J. (1975) *Biochem. Med.* **13**, 46-55.
17. Lis, J.T., Neckameyer, W., & Costlow, N. (1981) *Gene* **15**, 67-80.
18. Wiginton, D.A., Adrian, G.S., Friedman, R.L., Suttle, D.P., & Hutton, J.J. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 7481-7485.
19. Daddona, P.E. (1981) *J. Biol. Chem.* **256**, 12496-12501.
20. Nepveu, A., & Marcu, K.B. (1986) *EMBO J.* **5**, 2859-2865.
21. Bender, T.P., Thompson, C.B., & Kuehl, W.M. (1987) *Science* **237**, 1473-1476.
22. Green, P.J., Pines, O., & Inouye, M. (1989) *Ann. Rev. Biochem.* **55**, 569-597.
23. Munroe, S.H. (1988) *EMBO J.* **7**, 2523-2532.
24. Berkvens, Th.M., Schoute, F., van Ormondt, H., Kahn, P.M., & van der Eb, A.J. (1988) *Nucleic Acids Res.* **16**, 3255-3268.