Activity of the adenosine deaminase promoter in transgenic mice

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Received August 11, 1988; Revised and Accepted October 4, 1988

ABSTRACT

The promoter of the human gene for adenosine deaminase (ADA) is extremely G/C-rich, contains several G/C-box motifs (GGGCGGG) and lacks any apparent TATA or CAAT boxes. These features are commonly found in promoters of genes that lack a strong tissue specificity, and are referred to as "housekeeping genes". Like other housekeeping genes, the ADA gene is expressed in all tissues. However, there is a considerable variation in the levels of expression of the ADA protein in different tissues. In order to study the activity of the ADA promoter, transgenic mice were generated that harbor a chimeric gene composed of the ADA promoter linked to a reporter gene encoding the bacterial enzyme Chloramphenicol Acetyl Transferase (CAT). These mice reproducibly showed CAT expression in all tissues examined, including the hemopoietic organs (spleen, thymus and bone marrow). However, examination of the actual cell types expressing the CAT gene revealed the ADA promoter to be inactive in the hemopoietic cells. This was substantiated by a transplantation experiment in which bone marrow from ADA-CAT transgenic mice was used to reconstitute the hemopoietic compartment of lethally irradiated mice. The engrafted recipients revealed strongly reduced CAT activity in their hemopoietic organs. The lack of expression in hemopoietic cells was further shown to be correlated with a hypermethylated state of the transgene. Combined, our data suggest that the ADA promoter sequences tested can direct expression in a wide variety of tissues as expected for a regular housekeeping gene promoter. However, the activity of the ADA promoter fragment did not reflect the tissue-specific variations in expression levels of the endogenous ADA gene. Additionally, regulatory elements are needed for expression in the hemopoietic cells.

INTRODUCTION

Adenosine deaminase (ADA, EC 3.5-4.4) is an enzyme of the purine metabolism which catalyzes the deamination of adenosine and deoxyadenosine. In man, severe deficiency of ADA activity is associated with an autosomal recessive form of severe combined immunodeficiency (SCID) (1, 2). We and others have isolated sequences encoding human ADA and studies were initiated to investigate the basic molecular defects causing ADA deficiency (3-10). More recently, introduction of the ADA gene into retroviral vectors allowed experiments designed to test the possibilities for gene therapy in ADA⁻SCID patients (11-16).

Upon characterization of the human ADA gene, we also identified the promoter region which was found to contain some unique structural features: 1) it is extremely rich in G/C residues (82%); 2) it contains five G/C-box motifs (GGGCGGG) disposed in a strikingly symmetrical fashion, four within a 16-basepair (bp) repeat and one exactly in the center of this repeat; 3) it lacks the TATA and CAAT boxes which previously were thought to be characteristic for eukaryotic promoters transcribed by RNA-polymerase II (17). Currently similar features have been identified in promoter sequences of a variety of other genes that are not subject to a very tight regulation and that (like the ADA gene) are never completely switched off. These genes are often referred to as housekeeping genes. Examples are the genes encoding hypoxanthine phosphoribosyl transferase (HPRT), dihydrofolate reductase (DHFR), 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase, phosphoglycerate kinase (PGK), glucose 6-phosphate dehydrogenase (G6PD) and adenine phosphoribosyl transferase (APRT) (18-24). Unlike the expression of these other housekeeping genes, the human ADA gene is not expressed uniformly at low levels in all tissues (25). In contrast, there is a considerable variation in the ADA levels in different tissues ranging from 0.95 IU/g protein in red blood cells to 882 IU/g in thymus (26). Such a broad spectrum of ADA activity in different tissues was also found in mice, and recently it was shown that these differences in enzyme activity could be accounted for by the different levels of ADA mRNA (27, 28, 29).

Transgenic mice have been used to study the specificity of a variety of promoters in different cellular environments. Mostly these studies have focussed on promoter elements that direct transcription in a strict tissue specific fashion, resulting in transgene expression in selected cell types (see 30 for a review). In this study, we have investigated the activity of a promoter that was thought to lack such a specificity, the ADA promoter. Transgenic mice were generated that contain the promoter as well as the 5' untranslated region of the ADA gene fused to the gene encoding the bacterial enzyme Chloramphenicol Acetyl Transferase (CAT). Our data indicate that this promoter element can direct expression in most tissues. Surprisingly however, no transgene expression was detected in the hemopoietic cells.

MATERIALS AND METHODS

Construction of ADA-CAT fusion gene

pADA-CAT was constructed from pAMG1 (17), a pUC12-based ADA expression clone, which contains a 230 bp EcoRI-NcoI fragment from the human ADA gene carrying the 132 bp promoter as well as the leader sequences from the cap site

to the ADA initiation codon (at nt 95) which coincides with an NcoI site (ccATGg). This fragment was fused to a 1.6 kb HindIII-BamHI fragment derived from pSV2CAT (35) which contains the CAT gene followed by a 3' untranslated region of the SV40 T antigen. The latter includes the small t intron as well as transcription termination signals. The cloning procedure resulted in the destruction of the ADA initiation codon. In pADA-CAT the pUC12 polylinker was retained which allowed us to isolate the fusion gene by excising it with BamHI.

Transgenic mice

The DNA fragment containing the chimeric ADA-CAT gene (a 1.8-kb BamHI fragment from pADA-CAT) was isolated from low-melting agarose gels by the Elutip-d method (Schleicher and Schuell, Inc.). Samples (1 to 2 pl) of a solution of these DNA fragments (at a concentration of 1 µg/ml in 5mM Tris.HCl (pH 7.5), 0.1mM EDTA) were microinjected into fertilized mouse eggs obtained from superovulated C57BL/6 x BALB/c F₁ female mice mated to SJL males. The injected eggs were reimplanted into CD-1 pseudopregnant foster mothers. To identify transgenic mice, DNA was extracted from tail biopsies as described previously (31), digested with EcoRI and subsequently analysed by Southern blotting using ³²P-labeled CAT probe. We noted that all ADA-CAT transgenic mice expressed CAT in their tails. Therefore, this enabled us to identify transgenic mice in subsequent generations more rapidly by performing CAT assays on protein lysates from tail biopsies. HpaII methylation patterns of the transgenes in the various organs were determined by Southern blot analysis of HpaII- and MspI-digested DNA (32).

Stimulation of T- and B-cell populations

Con A and LPS stimulations were performed according to ref. 33. Fluorescence flow cytometric analysis indicated that the great majority of the Con A stimulated cells were positive for the Leukocyte-Common (L-C) antigen (99% in spleen, 95% in thymus). Light scatter profiles of the L-C Ag-negative- and the LPS-stimulated cells revealed that they were smaller than the L-C Ag-positive cells and therefore not of stromal origin.

CAT assays

To measure CAT activity, tissues were homogenized in 0.25 M Tris-HCl (pH 7.8) using an Ystral homogenizer. Homogenates were heated to 65°C for 5 min to inactivate inhibitory factors (34), cleared by centrifugation and assayed for CAT activity (35). Protein concentrations were determined according to Bradford (36).

Bone marrow transplantation

Bone marrow cells of transgenic mice were injected into lethally irradiated (10.5 Gy ¹³⁷Cs gamma rays whole body irradiation) C57BL/Rij x CBA/Rij

F₁ recipient mice. In this allogeneic setting transplantation of $1-5 \times 10^5$ bone-marrow cells generally resulted in the appearance of 5-15 spleen colonies in recipient mice, ten days after transplantation. Long-term survivors (more than 30 days after transplantation) were derived after injection of $1-5 \times 10^6$ bone marrow cells.

RESULTS

Generation of transgenic mice

In order to test the activity of the human ADA promoter, transgenic mice were generated that harbor a chimeric gene composed of the human ADA promoter fused to sequences encoding the bacterial enzyme CAT as a reporter molecule (pADA-CAT, see Fig. 1a). In pADA-CAT the CAT gene is preceded by a 230-bp EcoRI-NcoI fragment, encompassing the promoter of the human ADA gene, as well as all of its 5' untranslated sequences. The coding region is followed by a transcriptional termination signal provided by a 3' untranslated region derived from the SV40 T antigen (see Materials and Methods for the exact structure).

Transfection of this ADA-CAT fusion gene into a variety of cultured cell lines always resulted in CAT expression. In each case, the expression levels obtained with pADA-CAT were similar or higher as compared to those obtained with pSV2CAT. Cell lines tested include: mouse L-, NIH/3T3-, F9-, SD3 rat mamma tumor-, HepG2-, CV1-, cos- and HeLa cells (data to be published elsewhere). Furthermore, constructs containing additional upstream promoter sequences did not express significantly more CAT activity in mouse L- and HeLa-cells (Th. Perkvens, pers. comm.). These results suggested that the 230-bp DNA fragment contained the entire ADA promoter and might therefore direct expression into a wide variety of cell types in vivo as well.

Transgenic mice were produced by microinjection of a 1.8kb BamHI DNA fragment derived from pADA-CAT into the pronuclei of fertilized mouse eggs. Transgenic ADA-CAT mice were identified after hybridization of tail DNA with a probe for CAT (Fig. 1b). Tail DNA was digested with EcoRI which cuts only once in the injected 1.8 kb DNA fragment. As a result, tandemly integrated copies can be detected as 1.8-kb hybridizing fragments. This experiment identified transgenic mouse 91 which contains multiple integrated copies. Upon further breeding of mouse 91, tail-DNA analysis of the F₁ offspring revealed three different integration patterns. Representative mice exhibiting similar integration patterns are shown in Fig. 1b. This result is most likely due to the segregation of three loci, each containing multiple integrated ADA-CAT fusion

Figure 1

- A.** Structure of the ADA-CAT fusion gene. A 230-bp EcoRI-NcoI fragment, harboring both the ADA promoter and all of its 5' untranslated sequences was fused to the CAT gene followed by the 3'- untranslated region and polyadenylation signal of the SV40 T-antigen gene. Due to the cloning procedure, the NcoI site was destroyed.
- B.** Integration patterns of ADA-CAT DNA in transgenic mice. Tail DNA from transgenic mice was digested with EcoRI, separated by agarose gel electrophoresis, blotted onto nitrocellulose and screened with a ³²P-labelled CAT-probe. Mice representing the three different strains as represented by the different integration patterns were grouped.

genes and located on different chromosomes. Mice from two of these groups were bred further and yielded an F₂ generation in which the transgenes had integration patterns identical to the F₁ parental mice. Hence, two independent lines of transgenic mice were successfully established from a single founder.

Figure 2

CAT activity in various tissues from two ADA-CAT transgenic mice (nrs. 133 and 138). CAT assays were performed with 15 μ g of protein of each tissue lysate. For reference we used 0.5 units of pure enzyme (CAT) and lysis buffer without cell lysate (negative). After chromatography to separate the acetylated forms, autoradiography was performed for 48 hours and two weeks, respectively. Both exposures are shown. CM, unacetylated chloramphenicol, Ac1-M and Ac3-M chloramphenicol acetylated at the 1-hydroxyl and the 3-hydroxyl positions, respectively.

The Southern blot in Fig. 1b reveals that, in addition to the expected 1.8 kb fragment, the CAT probe also hybridizes to larger *EcoRI* fragments present in the DNA of the transgenic mice. These bands could represent cellular DNA sequences that flank the transgene. In addition, their presence could be the result of DNA methylation. This could also explain some of the differences in the intensity of hybridizing bands from animals of one strain.

CAT expression in the organs of ADA-CAT transgenic mice

To study the expression of the ADA-CAT transgene, CAT assays were performed on lysates of a variety of different organs obtained from 2-months-old transgenic mice (F_1 generation derived from mouse 91). Fig. 2 shows representative results of CAT assays, performed with lysates from mice 133 and 138. These two are representative for the two lineages of ADA-CAT containing mice, each displaying a different integration pattern (see also Fig. 1). CAT activity was detected in extracts from all organs tested. No great variations in expression level were apparent when comparing the different tissues except for liver, testis and bone marrow in which only low levels of CAT activity were

Figure 3

CAT activity in fractions of hemopoietic organs from ADA-CAT transgenic mice. Total spleen, thymus and bone marrow was forced through a sieve resulting in a single-cell suspension containing mainly the hemopoietic cells. In the case of spleen and thymus these cells were subsequently stimulated with mitogens to obtain pure populations of T-cells (Con A) or B-cells (LPS, spleen only). CAT assays were performed on lysates from total organs, tissue remaining on the sieve (not for bone marrow), the wash fluid, and the single-cell suspension, either directly after isolation or after stimulation with Con A (T-cells) or LPS (B-cells). Per assay 20 μ g (spleen and bone marrow) or 8 μ g (thymus) protein was used to determine the CAT activity in each fraction.

detected. In addition, skin tissue of mouse 133 also exhibited low CAT activity. It can be concluded that in both strains of mice CAT activity was detectable in a great variety of organs. Moreover, although the chimeric genes are located in two different chromosomal positions, their overall expression patterns were similar (with the exception of brain, trachea, eye, skin and heart).

Upon further breeding of the mice, the presence of ADA-CAT sequences always coincided with CAT-activity in the tissues of the progeny. Furthermore, it can be seen in Fig. 2 that higher relative levels of CAT expression were found in tissues of mouse 138 than in those of mouse 133. This trait was inherited, suggesting that the absolute levels of expression in the tissues are influenced by the integration site. In all subsequent studies we have used mice from the strain represented by mouse 138.

CAT activity in hemopoietic organs is not due to expression in the blood cells

Because of the potential applications of the human ADA promoter for expression of the ADA gene in hemopoietic cells we were interested to know which cell types actually express CAT activity in the hemopoietic organs of

Figure 4

Southern blot analysis showing successful reconstitution of hemopoietic tissues in mice transplanted with bone marrow from ADA-CAT transgenic mice. DNA was isolated from individual spleen colonies appearing 10 days post-transplantation, as well as from bone marrow, thymus and spleen from a long-term reconstituted animal. For reference, DNA was included from the tail of the bone marrow donor and the spleen from a non-transgenic mouse (-control). Southern blot analysis was performed on DNA digested with *EcoRI* using a CAT-specific probe.

the ADA-CAT mice. Therefore, we isolated spleen- and thymus-cell populations. Fractions were highly enriched for T- or B-cells by stimulation with either Concanavalin A (Con A) or lipopolysaccharide (LPS), respectively. Surprisingly, no CAT expression was found in these lymphoid cells derived from either spleen or thymus. Since CAT activity was readily detectable in extracts made from whole organs we performed a simple fractionation of the intact spleen and thymus into stromal and hemopoietic components and determined CAT activity in each of these fractions. The results of this experiment are depicted in Fig. 3. Upon forcing the spleen and thymus material through a sieve we could detect CAT activity only in the tissue remaining on the sieve and in the wash fluid. The latter was thought to be due to lysis of CAT-expressing cells. Histological examination of the remaining tissue on the sieve revealed that it consisted largely of connective tissue components. Hemopoietic cells were absent in this fraction. Similar results were obtained with a cell suspension from total bone marrow; CAT activity was detected in the wash fluid but not in the cell suspension representing largely hemopoietic cells. However, in this case no remaining material could be extracted from the sieve (see also Fig.

Figure 5

CAT activity in hemopoietic tissues from mice transplanted with bone marrow from ADA-CAT transgenic mice. CAT assays were performed on 20 μ g protein derived from the same engrafted tissue as shown in Fig. 5. For comparison, CAT assays from various organs of the transgenic donor mouse were included. For reference, 0.5 units of pure CAT enzyme (CAT) and lysis buffer without cell lysate (-) were included.

3). Finally, the red blood cells were also found to be completely devoid of CAT activity. Combined, these data led us to suspect that the ADA promoter fragment was inactive in possibly all the hemopoietic cells of the transgenic mice. To substantiate this, we studied CAT expression in hemopoietic tissues of mice after reconstitution with bone marrow from transgenic mice. A successful engraftment would result in chimeric mice in which the hemopoietic cells carry ADA-CAT sequences whereas the stromal components do not. Successful grafting was monitored by Southern blot analysis of DNA from the hemopoietic organs of mice that had received a bone marrow transplantation. Fig. 4 shows that ADA-CAT sequences were detected in the spleen, thymus and bone marrow of a reconstituted mouse 33 days after transplantation. Similarly, DNA from spleen colonies that appeared 10 days after transplantation carried the transgene. Despite successful engraftment of the transgenic bone marrow, CAT activity was not detected in lysates from the inspected spleen colonies, and not in the bone marrow and thymus of the long-term reconstituted mice (see Fig. 5). Quantification of the CAT assay revealed the cells of the chimeric spleen to express 80-fold less CAT than the spleen cells of the donor. In conclusion, CAT expression in the hemopoietic organs of the transgenic donor mice is

Figure 6

Methylation patterns of organs from ADA-CAT transgenic mice. DNA was isolated from the cell types indicated and restricted with HpaII (H) or MspI (M), respectively. Southern blot analysis was performed with a radio-labeled CAT-specific probe (1.6 kb HindIII-BamHI fragment from pSV2CAT).

restricted to non-hemopoietic cells. Apparently the ADA promoter sequences tested are insufficient to promote expression in hemopoietic cells.

The non-expressing hemopoietic cells carry hypermethylated ADA-CAT sequences

Several previous studies have indicated that non-expressing genes are often hypermethylated and vice versa. Therefore, we have investigated whether in our ADA-CAT transgenic mice such a relationship also exists. DNA from cell types that did or did not express the CAT gene was digested with the enzymes HpaII and MspI. Both enzymes cleave the sequence CCGG, but the methylated version of the palindrome (C^{me}CGG) can only be cleaved by MspI and not by HpaII. The ADA-CAT fusion gene contains eleven MspI sites of which seven are present in the 230 bp ADA promoter and leader fragment. Southern blots revealed six MspI fragments hybridizing to the CAT probe (see Fig. 6). Their respective sizes were estimated to be 0.48, 0.59, 0.91, 1.12, 1.55 and 3.3 kb. Of these, only the 3.3-kb fragment was present in HpaII-digested DNA from the non-expressing spleen, thymus and bone-marrow suspension cells. The other CAT sequences all remained in the high-molecular weight fraction, indicating that most of the MspI sites in the transgenes are methylated. For comparison, we have also analysed DNA from tissues that express CAT at considerable levels

Figure 7

Methylation patterns of the ADA-CAT transgene in hemopoietic organs after bone marrow transplantation. DNA was isolated from bone marrow, spleen, thymus and spleen colonies of engrafted recipients, after transplantation with bone marrow from a transgenic mouse (see also Figs. 5 and 6). The Southern blot shows HpaII (H)- and MspI (M)-digested DNA probed with a CAT-specific probe (1.6 kb HindIII-BamHI fragment from pSV2CAT). Since all detectable HpaII sites of the transgene in testis DNA are unmethylated, it is included as a positive control.

like eye and brain, as well as testis in which transgenes are often found to be undermethylated (37, 38). Digestions of testis DNA with MspI and HpaII resulted in identical patterns indicating that all the analyzed MspI sites in the transgenes are unmethylated. In DNA from brain and eye, the 0.91- and 3.3-kb fragments were not detected after HpaII cleavage whereas the 0.48-, 0.59-, 1.12- and 1.55-kb fragments were still detectable, indicating a much lower degree of methylation as compared to tissues that do not express ADA-CAT.

The above data suggest that a correlation exists between suppression of the ADA-CAT-gene and methylation of MspI sites. This correlation was also found in hemopoietic tissues of mice reconstituted with bone marrow from ADA-CAT transgenic mice. As shown in Fig. 7, most of the CAT sequences remain in the high molecular weight fraction after HpaII digestion. DNA isolated from spleen colonies displayed an intermediate methylation pattern. This could be due to the relatively high proportion of dividing cells in such colonies.

DISCUSSION

The promoter of the human gene for ADA is active in all tissues and contains features that are commonly found in promoters for housekeeping genes (i.e., G/C-rich, several G/C-box motifs and no apparent TATA or CAAT boxes; see 25 for a review). To study the activity of this promoter in a variety of cellular environments we have produced transgenic mice that contain 132 bp of the 5' flanking sequences of the human ADA gene fused to a reporter gene encoding CAT. These mice expressed CAT activity in all tissues tested, which is consistent with the known activity of the human and mouse ADA gene. Therefore, in this respect the 230-bp DNA fragment encompassing the human ADA promoter sequences investigated seemed sufficient to direct ubiquitous gene expression of a reporter gene. We believe that this activity is a general characteristic of the promoter fragment since similar expression patterns were found in two different strains of ADA-CAT transgenic mice as well as in mice that harbor another fusion gene in which the same ADA promoter sequences were placed in front of the human ADA cDNA (to be published elsewhere). Unexpectedly, crude fractionation of hemopoietic tissues (spleen, thymus, bone marrow) revealed that hemopoietic cells were devoid of CAT activity. The CAT activity detected in these tissues was restricted to the stromal cells. This was substantiated by a bone marrow transplantation experiment. Bone marrow from transgenic mice was transplanted into lethally irradiated non-transgenic recipients. Upon engraftment the recipient mice are chimeric, their hemopoietic cells being derived from hemopoietic stem cells of the transgenic donor mouse. Essentially no CAT activity could be detected in the reconstituted organs which led us to the conclusion that the 132-bp human ADA promoter fragment cannot direct expression in hemopoietic cells. We did, however, detect a very low level of CAT activity in the spleen (but not in thymus or bone marrow) of one mouse that had received a bone marrow transplant. It can therefore not be excluded that a minor subpopulation of hemopoietic cells in these tissues still expresses the CAT gene. To test this, we attempted to detect the marker gene product using immunohistochemistry with a polyclonal rabbit anti-CAT antiserum. We were unable to detect CAT protein using this strategy (not shown). Therefore, we are currently producing new lines of transgenic mice carrying a marker gene that is more easily detectable at low levels using standard histochemical techniques (lacZ).

Since the ADA promoter, as well as most other housekeeping promoters, has a significantly higher CpG/GpC doublet ratio than that found in the remainder of the genome, it has been proposed that CpG-islands are often found in parts

of the eukaryotic genome where the continuous binding of transcription factors provides a lasting protection against cytosine methylation (27, 39). Furthermore, for many genes a causal relationship has been found between hypermethylation and gene inactivity, and vice versa (reviewed in 40). We therefore determined the methylation patterns of the transgenes in tissues in which the reporter gene was either expressed or not expressed. Our data clearly indicate that in hemopoietic cells the transgenes are largely hypermethylated, whereas in tissues where CAT activity was detected, the transgenes were found to be undermethylated. Treatment of the hemopoietic cells with the demethylating agent 5-azacytidine will establish whether this reflects a causal relationship.

In summary, our data show that the 132-bp ADA promoter fragment can direct expression of a reporter gene in a great variety of different cell types as can be expected for a promoter of a typical housekeeping gene. Gene expression did, however, not always reflect the specific tissue distribution of endogenous ADA (high in hemopoietic organs and alimentary tract, low in kidney, lung, liver and brain) and surprisingly, expression was absent in blood cells. This is unexpected since apparently in ADA-deficient patients the only cell types that absolutely require ADA activity are of hemopoietic origin (8). We would therefore like to propose that hemopoietic ADA expression is controlled by a thusfar unidentified, distinct regulatory element, which is not included in the transgenes described in this paper. Our findings that the 132-bp ADA promoter DNA fragment is not active in the hemopoietic T- and B-cell lineages is of considerable importance for future experiments aimed at reconstitution of ADA gene activity in the hemopoietic system using retroviral vector technology. In particular, retroviral vectors to be used in gene therapy for ADA⁻SCID patients should preferably express ADA in the T-cell lineage. Hence, the use of human ADA promoter sequences in retroviral vector systems (12) should await the identification of the relevant regulatory elements.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. Martin Kast for his technical advise concerning the T- and B-cell stimulation, Dr. Jan Bauman for the flow cytometric analysis, Dr. Gary McMaster for providing the polyclonal rabbit anti-CAT antiserum, Theo Berkvens for sharing unpublished data, Jan van der Brugge for breeding the transgenic mice, Drs. Ron Evans and Inder Verma for their support in the production of the transgenic mice and Hans van Ormondt for critically

reading this manuscript. This work was supported in part by the Netherlands organization for Scientific Research (NWO).

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