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Adenosine deaminase: characterization and expression of a gene with a remarkable promoter

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Cosmid clones containing the gene for human adenosine deaminase (ADA) were isolated. The gene is 32 kb long and split into 12 exons. The exact sizes and boundaries of the exon blocks including the transcription start sites were determined. The sequence upstream from this cap site lacks the TATA and CAAT boxes characteristic for eukaryotic promoters. Nevertheless, we have shown in a functional assay that a stretch of 135 bp immediately preceding the cap site has promoter activity. This 135-bp DNA fragment is extremely rich in G/C residues (82%). It contains three inverted repeats that allow the formation of cruciform structures, a 10-bp and a 16-bp direct repeat and five G/C-rich motifs (GGGCGGG) disposed in a strikingly symmetrical fashion. Some of these structural features were also found in the promoter region of other genes and we discuss their possible function. Knowledge of the exact positions of the intron-exon boundaries allowed us to propose models for abnormal RNA processing that occurs in previously investigated ADA-deficient cell lines.

Key words: adenosine deaminase/SCID/gene structure/minigene expression/G+C-rich motifs

Introduction

Adenosine deaminase (ADA, EC 3.5.4.4) catalyzes the deamination of adenosine and deoxyadenosine. The catalytic activity of human ADA resides in a polypeptide with a mol. wt. of 42 000 as measured by SDS-polyacrylamide gel electrophoresis (Daddona and Kelley, 1977; Valerio *et al.*, 1983). The enzyme is encoded by a single genetic locus on the long arm of chromosome 20 (Tischfield *et al.*, 1974). There is a great variation in the ADA activity in different tissues, ranging from 0.95 IU/g protein in red blood cells to 882 IU/g in thymus (J. ten Kate, personal communication). Recent studies show that the expression of the ADA gene in several differentiation-inducible cell lines is inversely correlated with the degree of their maturation (our own unpublished results). However, in none of the tissues investigated thus far was the ADA gene found to be completely switched off.

Severe deficiency of ADA activity in man is associated with an autosomal recessive form of severe combined immunodeficiency (SCID) disease (Giblett *et al.*, 1972; Thompson and Seegmiller, 1980). The metabolic effects of ADA deficiency and its influence on the immune system have been intensively studied (Martin and Gelfand, 1981). Recently, we and other groups have isolated sequences encoding human ADA (Valerio *et al.*, 1983; Orkin *et al.*, 1983; Wiginton *et al.*, 1983). The availability of these clones allowed studies of the structure and expression of the ADA gene in wild-type and ADA$^-$SCID cells (Valerio *et al.*, 1984a; Daddona *et al.*, 1984; Adrian *et al.*, 1984).

Northern blot analysis reveals a major ADA mRNA species of ~1.5 kb, that has been shown to code for the 42 000 mol. wt. ADA protein by *in vitro* translation of size-fractionated RNA (Valerio *et al.*, 1983). In addition, a minor hybridizing RNA species of ~5 kb is sometimes observed. Hybridization experiments showed, however, that this RNA has only limited homology with the 1.5-kb species (Daddona *et al.*, 1984; our unpublished observation). Sequence analysis of a 1462-bp ADA cDNA clone pLL (Valerio *et al.*, 1984b), revealed an open reading frame with a coding capacity of 40 762 daltons. By gene transfer experiments, we have shown that this sequence codes for an active enzyme with the physico-chemical properties of human ADA (Valerio *et al.*, 1984b). It was therefore concluded that this cDNA clone contains the complete coding sequence of human ADA. We describe here the isolation and characterization of the complete gene for normal human ADA. It was found to contain 12 exons distributed over 32 kb of genomic DNA. Furthermore, its promoter region contains some noteworthy structural elements that are discussed.

Results

Isolation and characterization of the ADA gene

A cosmid library constructed from normal human placental DNA was screened with our ADA cDNA clone pADAc2 (Valerio *et al.*, 1983). Seven overlapping cosmid clones were isolated, spanning in total 66 kb. Three of these were studied further; in Figure 1 their relative positions, as well as restriction maps for *Eco*RI, *Sac*II, *Sal*I and *Xho*I are shown. Exon-containing restriction fragments were identified by using fragments from the nearly full-length ADA cDNA clone pLL (Valerio *et al.*, 1984b) as probes on Southern blots of cosmid DNA cut by several restriction enzymes. The exon-containing restriction fragments indicated in Figure 1, were subcloned into convenient vectors. From these subclones, the fine structure was determined. To define the boundaries of the exon blocks, we performed sequence analysis either directly by chemical degradation (Maxam and Gilbert, 1980) or, after further subcloning into M13-phage, by chain termination with dideoxy triphosphates (Sanger *et al.*, 1977; Messing and Vieira, 1982). The results are shown in Figures 1 and 2. The first exon is located on a 4.8-kb *Eco*RI fragment and encodes the previously published 61 nucleotides of 5'-untranslated RNA plus the N-terminal 10 amino acids. A subclone containing this 4.8-kb *Eco*RI fragment was designated pPRE. The 12th exon encompasses the four C-terminal codons as well as the 3'-untranslated region with the polyadenylation signal. The other 10 exons are evenly distributed over the genome with the exception of the second exon which is surrounded by two large introns of ~15 and 7 kb. All sequences around the intron-exon boundaries are consistent with the

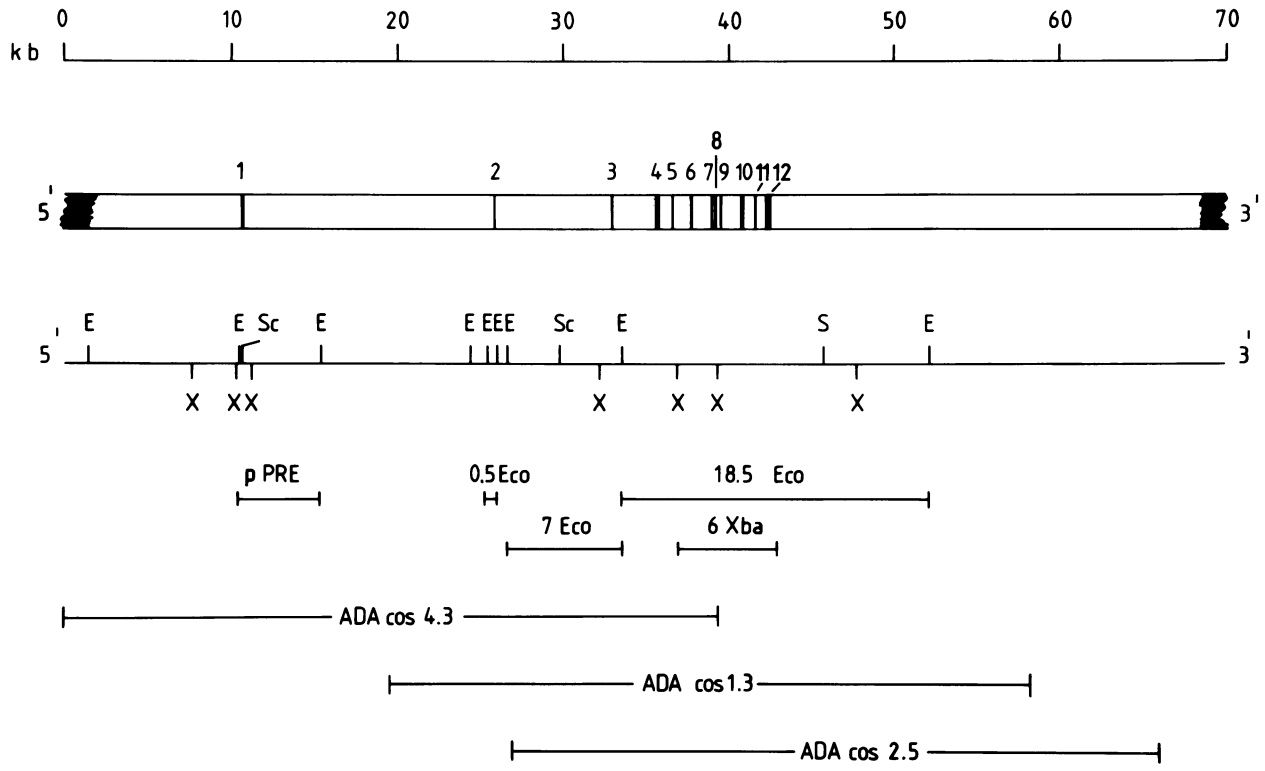

Fig. 1. A physical map of the ADA gene. The entire human ADA gene is shown as the bar at the top of the diagram. Exons 1 – 12 are denoted by solid boxes, introns by open boxes. Below this bar, a physical map for the enzymes *Eco*RI (E), *Sac*II (Sc), *Sal*I (S) and *Xho*I (X) is drawn. Three overlapping cosmid clones used to characterize the gene are indicated at the bottom of the figure, as well as the subcloned genomic fragments. The *Eco*RI and *Xba*I fragments were subcloned in the vectors pUC12 and pUC13.

reported consensus splice signals (Breathnach and Chambon, 1981). Sequence analysis of the first 163 bp of genomic DNA immediately upstream from the 5′ terminus of our cDNA showed that it is extremely rich in G/C (82%). This tract contains three inverted repeats and two direct repeats of 16 and 10 bp (see Figure 2). The inverted repeats would allow the formation of cruciform structures, two of which are mutually exclusive; the most stable ones ($\triangle G = -42.4$ and -23.2 kcal/mol for the individual hairpins; Tinoco *et al.*, 1973) are depicted in Figure 3B. Another noteworthy feature of this region is the presence of five GGGCGGG boxes in a symmetrical fashion; two, partially overlapping in the 16-bp repeat segments and one exactly in the middle of them (see Figure 2).

Characterization of the 5′ end of the ADA messenger

In Figure 3A, a fine map of the 5′ end of the gene, which is contained in plasmid pPRE, is shown. To estimate the distance between the cap site and a *Nae*I site which is located 5 residues 3′ from the beginning of our cDNA sequence, we have performed a primer extension experiment. A 5′-labeled 119-bp *Bam*HI-*Nae*I fragment from cDNA clone pLL was hybridized to ADA mRNA from MOLT-4 cells. This hybrid was then used to prime DNA synthesis by reverse transcriptase. This resulted in an elongated product of 154 nucleotides (not shown), implying that elongation by reverse transcriptase stops approximately 30 nucleotides upstream from the 5′ end of our cDNA sequence. This is in good agreement with the results of Daddona *et al.* (1984), who also performed a primer extension experiment on the ADA messenger. However, our sequence data on the genomic DNA revealed an inverted repeat that, when present in the RNA, could lead to a hairpin structure at a position 28 bp in front of the first residue of the cDNA sequence (see Figure 3B). Therefore, we could not exclude the possibility that the length of the elongation product indicated the position of a strong stop rather than the 5′ end of the messenger. To clarify this, we have performed an S1 nuclease experiment. As a probe, we used an internally labeled single-stranded 103 nucleotide long *Nco*I-*Fnu*DII fragment (see Figure 3 and also Materials and methods). This DNA fragment was chosen because it contains no inverted repeats that could lead to the formation of secondary structures. The ^{32}P-labeled probe was hybridized to MOLT-4 mRNA and incubated with S1 nuclease. The protected DNA segment was run on a sequencing gel alongside a Maxam-Gilbert sequencing ladder of a 230-bp *Nco*I-*Eco*RI fragment from pPRE that was 5′ end-labeled at the same *Nco*I site that bordered the S1 probe. In Figure 4 it can be seen that there are four residues between the termini of the intact S1 probe and the major S1-resistant fragment. From this we conclude that the actual 5′ end of exon 1 is at the A/T pair, 5 bp downstream from the *Fnu*DII cleavage site. It should, however, be mentioned that a comparison between the positions of the products in the S1 lanes (lanes 1,2 and 3 in Figure 4) and the sequencing ladders shows that the mobility of the enzyme products is the equivalent of two nucleotides faster than expected. We have no explanation for this phenomenon. Identical results were obtained when this S1 nuclease experiment was performed with a 5′ end-labeled S1 probe (not shown). The distance between the putative cap site and the beginning of our cDNA sequence is 33 bp, which is in good agreement with the primer extension experiments. However, the region upstream from the putative cap site lacks the characteristic TATA and CAAT boxes usually present in eukaryotic promoters.

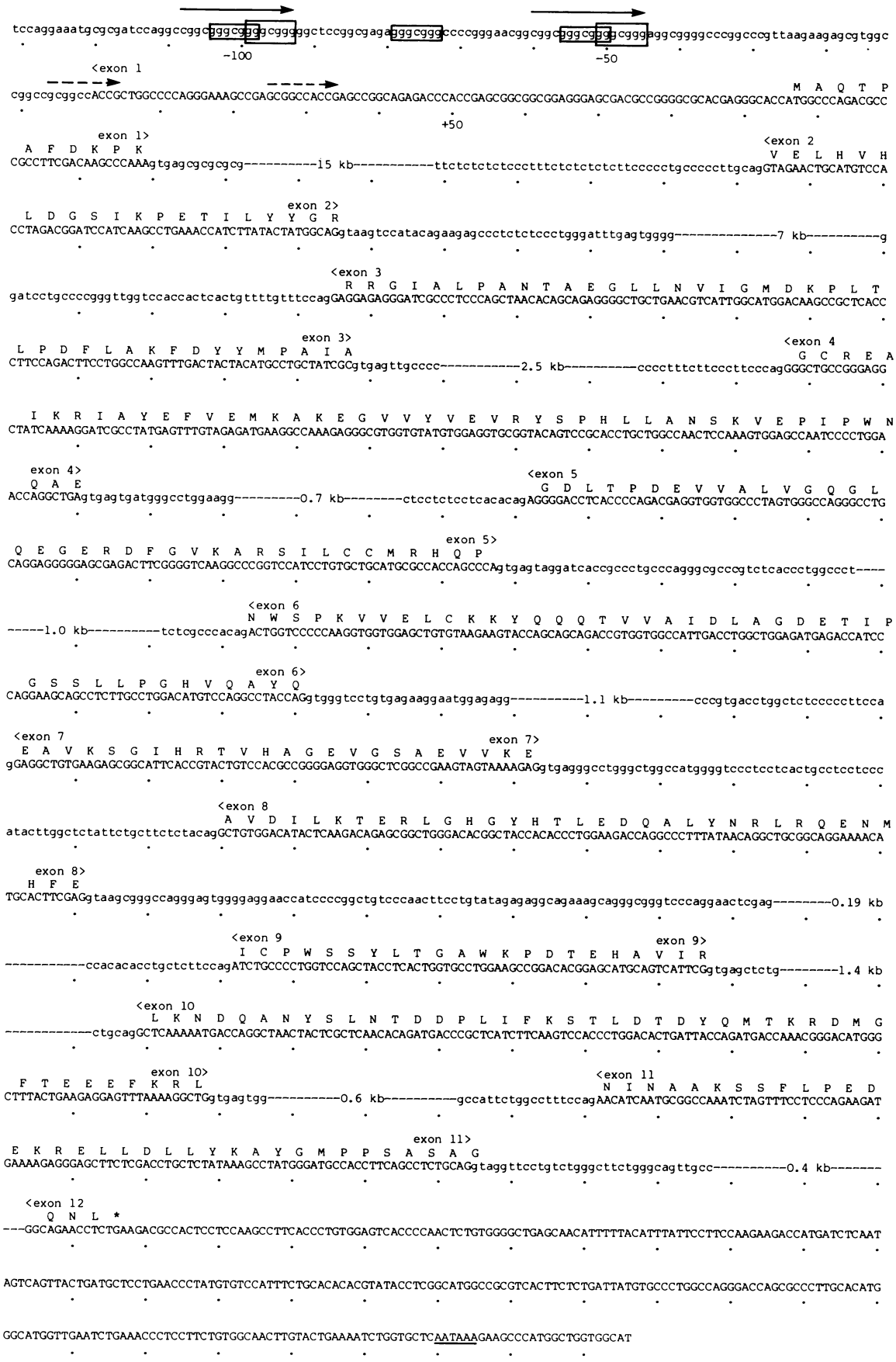

Fig. 2. The nucleotide sequence of the 12 exons in the human ADA gene including the intron-exon junctions and 135 bp of upstream sequences. Exon sequences are in upper case. The deduced amino acid sequence is indicated above the nucleotides in one-letter code. Arrows above the sequence indicate the positions of the 16- and 10-bp direct repeat elements. G/C-rich motifs are boxed.

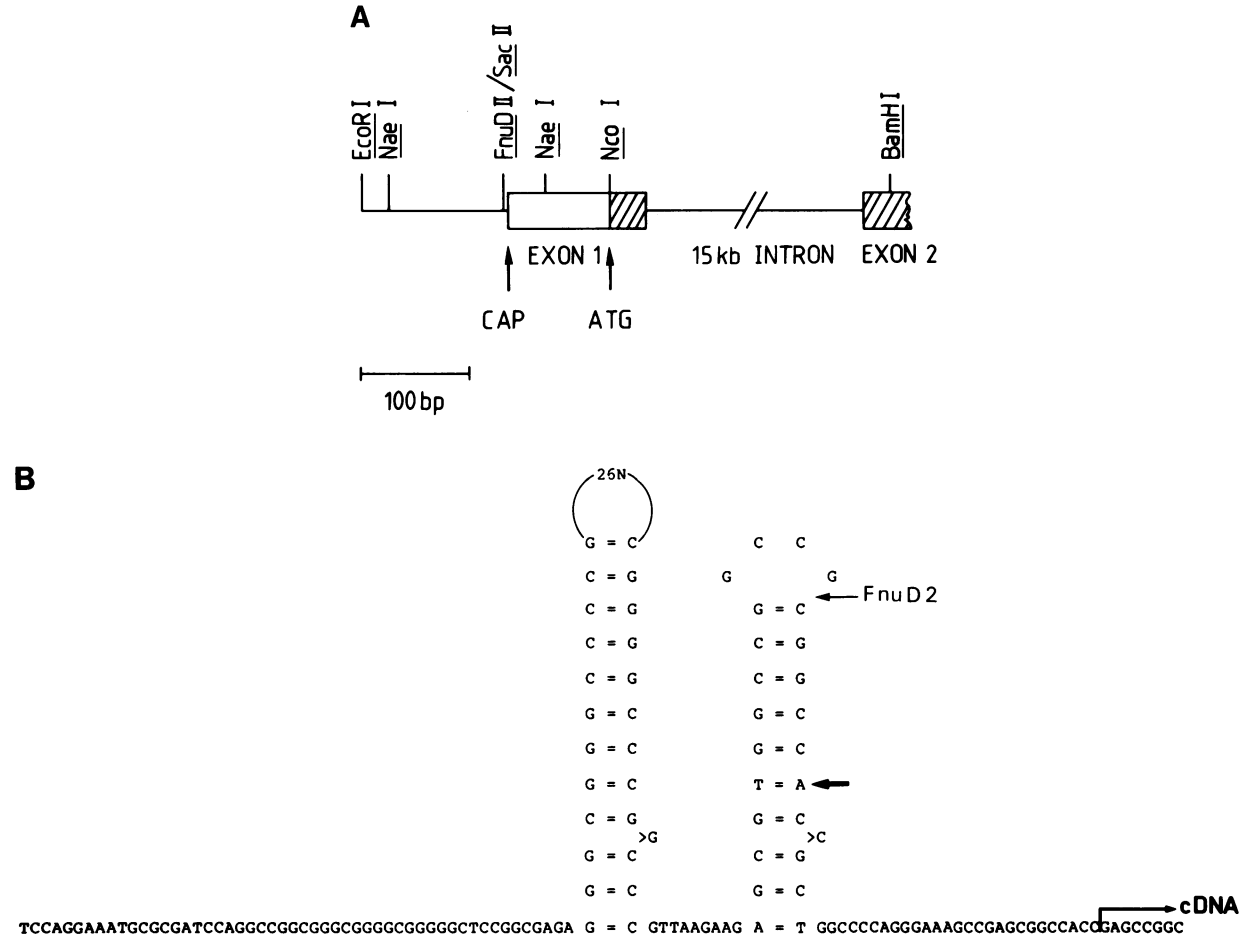

Fig. 3. (A) A fine map of the 5′ end of the ADA gene. The non-coding region is denoted by an open box, the coding sequences by hatched boxes. The positions of the cap site (cap) and the translation initiation codon (ATG) are indicated. **(B)** Two potential alternative secondary sructures in the promoter region of the ADA gene. The arrow indicates the putative cap site; the position of the *Fnu*DII cleavage site that bordered the S1 probe (see Results) is also indicated. A third alternative structure that is excluded by the formation of the most 3′ hairpin depicted in this figure is not drawn.

Expression of an ADA minigene in mouse cells

Further proof that the 5′ end of the ADA gene is located within the 230-bp *Eco*RI-*Nco*I fragment was obtained by assigning promoter activity to this fragment. This was done by gene transfer experiments on cultured mouse cells using an ADA minigene construct (see Figure 5). The construct contains the 230-bp *Eco*RI-*Nco*I fragment which harbors 135 bp of DNA upstream from the putative cap site. In addition, this fragment contains the sequence that codes for the entire 98-nucleotide 5′-untranslated region, since the *Nco*I site straddles the initiation codon for ADA. This *Eco*RI-*Nco*I promoter fragment was placed upstream from an *Nco*I-*Eco*RI fragment isolated from the ADA expression plasmid pMAMD (Valerio *et al.*, 1984b). The latter comprises the complete coding sequence for human ADA; starting at the aforementioned *Nco*I site (ccATGg), and followed by a 3′-untranslated region derived from the hepatitis B virus surface antigen gene. A three-part ligation was used to insert this minigene into the *Eco*RI site of pUC13 (Messing and Vieira, 1982; see also Figure 5). The resulting clone was designated pAMG1. As a control, we deleted the *Eco*RI-*Nco*I promoter fragment from pAMG1 and obtained pAMG1C (see Figure 5). By sequence analysis we confirmed that the ATG initiation codon was still present in pAMG1C.

The ability of our minigene constructs to express ADA activity was tested by DNA-mediated gene transfer into mouse L cells (see Figure 6). Two days after transfer, the cells were harvested and cell lysates were subjected to electrophoresis on cellulose acetate gels in order to separate the mouse and human ADA isozymes. After electrophoresis, the gels were stained *in situ* for ADA activity. Transfection of mouse cells with pMAMD and pAMG1 resulted in a band of human ADA activity on a mouse background (lanes 2 and 3). Cells transfected with control plasmid pAMG1C gave no detectable ADA signal co-migrating with human ADA (lane 4). From these results we conclude that the 135-bp DNA segment located 5′ from the putative cap site of the ADA gene contains promoter activity.

Discussion

This work describes the detailed structure of the human ADA gene. The gene consists of 12 exons that span 32 kb. We have determined the 5′ end of the ADA mRNA by using both primer extension and S1 nuclease mapping assays. Subsequently, 135 bp of upstream sequences were placed in front of the coding sequence of human ADA in a minigene construct. The ability of this construct to transfer human ADA activity to mouse cells provides functional proof that initiation of transcription can occur in this region.

The sequence of the human ADA promoter region is extremely G/C-rich (82%). It reveals no classical TATA box, which is typically located between positions −20 to −30 and

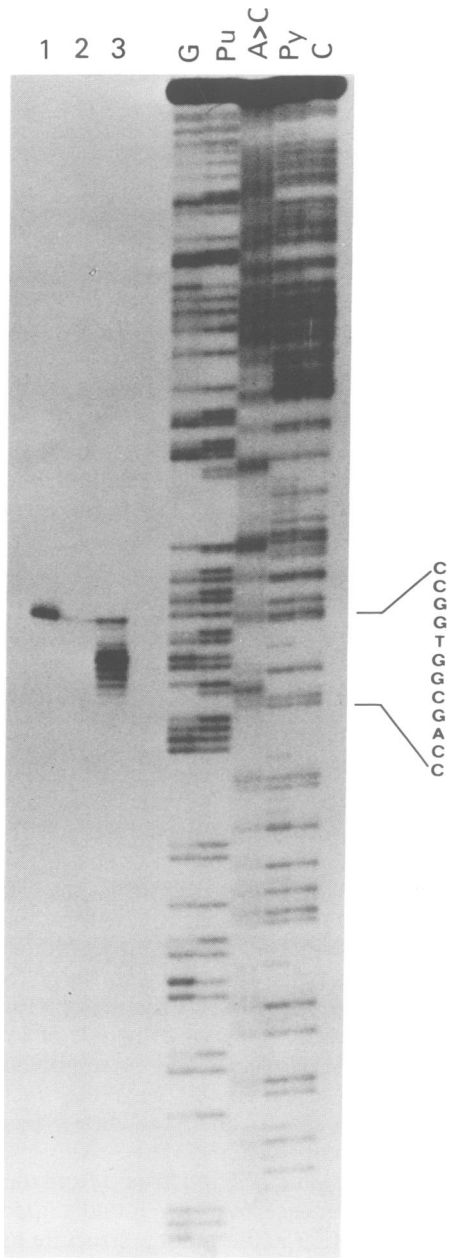

Fig. 4. S1 nuclease analysis of the 5′ end of the ADA mRNA. An internally labeled 103 nucleotide long *Fnu*DII-*Nco*I fragment was hybridized to MOLT-4 RNA, digested with S1 nuclease and fractionated on a gel alongside a sequencing ladder of an *Eco*RI-*Nco*I fragment that was 5′ labeled at the same *Nco*I site that bordered the S1 probe. **Lane 1:** the *Nco*I-*Fnu*DII S1 probe; **lane 2:** S1 nuclease analysis control experiment with tRNA; **lane 3:** S1 nuclease analysis with MOLT-4 RNA.

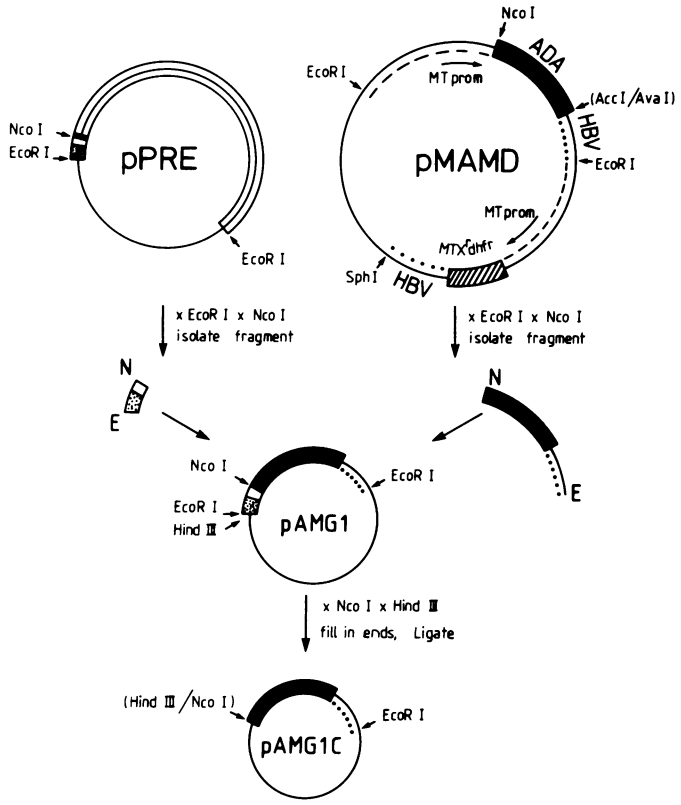

Fig. 5. Construction of the ADA minigene. A 230-bp *Eco*RI-*Nco*I fragment, containing the 5′-untranslated region from ADA (open bar) and 135 bp of upstream sequences (shaded bar), was isolated from pPRE and ligated to an *Nco*I-*Eco*RI fragment from pMAMD which contains the complete coding sequence for ADA (solid bar) followed by the 3′-untranslated region of the hepatitis B virus surface antigen gene (dotted line). This chimaeric molecule was ligated in the *Eco*RI site of pUC13 and used to transform *Escherichia coli* DH1 in order to obtain pAMG1. pAMG1 was subsequently cut with *Hind*III and *Nco*I, treated with Klenow's fragment of DNA polymerase to obtain blunt ends, religated, and used to transform *E. coli* DH1. The resulting minigene which lacks a eukaryotic promoter region was designated pAMG1C. Vector sequences are indicated as single lines.

has the consensus sequence TATA$^{A}_{T}$A$^{A}_{T}$ (Breathnach and Chambon, 1981), unless the sequence TTAA (position −27) functions as such. The so-called CAAT box which is often found ~80 bp in front of eukaryotic genes (Benoist *et al.*, 1980) is also absent. Recently, the promoters of two other genes have been described that are also high in G/C content and lack the characteristic TATA and CAAT boxes. These genes code for hypoxanthine phosphoribosyl transferase (HPRT, Melton *et al.*, 1984) and 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase (Reynolds *et al.*, 1984). Interestingly, both these enzymes are considered to be 'housekeeping' enzymes that, like ADA, are expressed at low levels in a great variety of tissues. Therefore, our data support the suggestion made by Melton *et al.* (1984) and Reynolds *et al.* (1984) that the absence of the TATA and CAAT boxes is common for housekeeping genes.

We have compared the primary and secondary structures of the ADA promoter with those of some other genes. As can be seen in Figure 7, homologies can be found in the DNA sequence of the ADA promoter and those of the SV40 early and late regions, the mouse genes for HPRT and the p53 tumor antigen, the chicken gene for α2 type I collagen and the tk genes from chicken and herpes simplex virus (for references, see legend to Figure 7). The presence of these stretches of homology suggests common functions in the various promoters. One of the most salient features of the ADA promoter is the presence of five GGGCGGG boxes in a symmetrical fashion, four within a 16-bp repeat and one exactly in the center of this repeat. These boxes are homologous to the so-called G/C-rich motifs (CCGCCC) of which six are present in the 21-bp repeat region of the SV40 promoter region. The functional relevance of these G/C-rich motifs has been studied extensively, and they appear to be essential for the efficient transcription of early and late SV40 gene products, both *in vivo* and *in vitro* (Everett *et al.*, 1983; Baty *et*

al., 1984; Vigneron *et al.*, 1984). Furthermore, a transcription factor, Spl (Dynan and Tjian, 1983a), has been shown to bind to a region in the 21-bp repeat that contains G/C-rich motifs (Dynan and Tjian, 1983b). Interestingly with respect to the ADA promoter, the effects of these G/C-rich motifs are independent of their orientation and it has been hypothesized that the presence of these elements facilitates the recognition of a 'weak TATA box' (Vigneron *et al.*, 1984). G/C-rich motifs with similar functions have also been identified in the promoters of two herpes virus genes encoding thymidine kinase and glycoprotein D (McKnight and Kingsbury, 1982; McKnight *et al.*, 1984; Everett, 1984) and their presence was noticed in the promoters of the housekeeping genes HPRT and HMG CoA reductase (Melton *et al.*, 1984; Reynolds *et al.*, 1984). Most of the cellular promoters that have been studied thus far are subject to strong regulation and are often completely 'switched off' in specific tissues. It is possible that promoters of housekeeping genes such as ADA, and some viral genes, have a different organization. If so, one of the features of these promoters are the G/C-rich motifs like the GGGCGGG boxes in the ADA promoter region. These elements could act as binding sites for a transcription initiation factor like Spl that is specific for these types of promoters as suggested by Dynan and Tjian (1983a). In this respect, it would be interesting to investigate whether the ADA promoter region requires 'Spl-like transcription factors' for its activity *in vitro*.

The positions of the two inverted repeats in the ADA promoter that allow the formation of cruciform structures were also compared with the positions of these elements in other promoters. In the case of the ADA promoter, the initiation of transcription takes place at the 3′ stem of a proposed cruciform structure, while the TATA box is located just upstream from this structure (see Figure 3B). A similar configuration has been reported for several other eukaryotic genes, such as: the E1A and major late transcription units of adenovirus types 5, 7 and 12, rabbit β-globin, *Bombyx* fibroin, chicken ovalbumin, chicken α2 type I collagen and maize zein (Engler *et al.*, 1981; Vogeli *et al.*, 1981; Langridge and Feix, 1983). The homology in the position of the secondary structures in these promoters probably indicates their relevance. Their function, however, remains to be elucidated.

Knowledge of the exact position of the intron-exon boundaries of the ADA gene allows us to propose a structure for the anomalously processed mRNAs of some cell lines derived from ADA-deficient humans. To date, two models for the genetic basis of ADA deficiency have been put forward: (i) Point mutations in the coding region of the ADA gene that cause amino acid substitutions which render the ADA protein either unstable or catalytically inactive (Valerio *et al.*, 1984a; Valerio *et al.*, in preparation); (ii) small deletions or insertions in the ADA messenger that are probably caused by aberrant RNA processing as described by Adrian *et al.* (1984). These authors have performed S1 nuclease analysis of hybrids between a full-length ADA cDNA and corresponding mRNA from ADA-deficient cells. In this way, they were able to detect an insertion in 10% of the ADA messengers from the partially ADA-deficient cell line GM3043. This insertion was located approximately at a position 280 bp from the 5′ end of their ADA cDNA sequence. We have found a large intron of ~2.5 kb at that position in the genome structure between exons 3 and 4. Since there is no difference detectable between the length of the normal and GM 3043 ADA mRNA on Northern blots, it is possible that a small portion of this intron is still contained in the mature messenger in cell line GM3043. A similar observation had already been made by Wiginton *et al.*

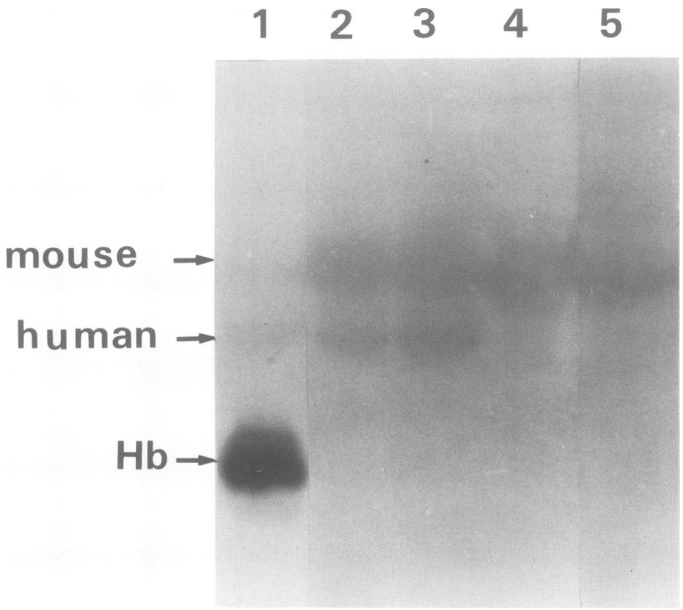

Fig. 6. Analysis of mouse cells for the presence of human ADA. Mouse L cells were transfected with pMAMD **(lane 2)**, pAMG1 **(lane 3)**, and pAMG1C **(lane 4)**. Two days after transfection lysates were prepared which were fractionated on cellulose acetate gels. Human red blood cells **(lane 1)** and untransfected mouse L cells **(lane 5)** were used as controls to indicate the mobilities of the human and mouse isozymes in this system. After electrophoresis the gel was stained *in situ* for ADA activity. Hb indicates the position of hemoglobin, present in the red blood cell lysate.

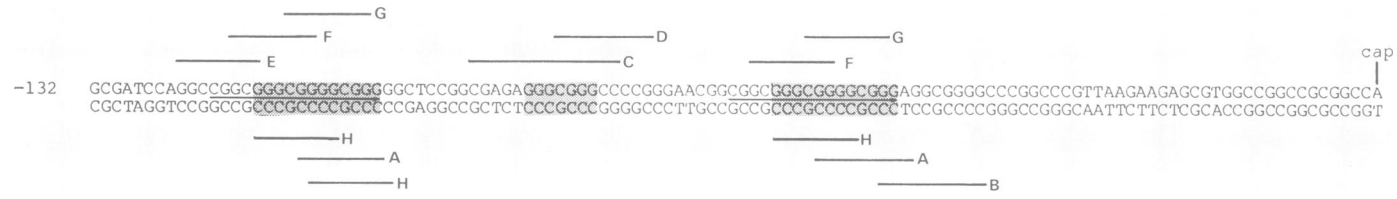

Fig. 7. Comparison between the nucleotide sequence of the ADA promoter region and that of other genes. The 132 bp of sequence upstream from the putative transcription initiation site exhibits homologies with the promoter regions of: **(A)** SV40 DNA (74–83, 95–104, 21-bp repeat; Fiers *et al.*;, 1978); **(B)** mouse p53 tumor antigen gene (−257 to −266; Bienz *et al.*, 1984); **(C)** mouse HPRT gene (−36 to −49, Melton *et al.*, 1984); in HPRT DNA, this sequence is in the center between two decamer repeats; **(D)** chicken α2 type I collagen gene (−105 to −96; Vogeli *et al.*, 1981); **(E and F)** chicken tk gene [162–170 **(E)**, 218–225 **(F)**; Merrill *et al.*, 1984]; **(G and H)** HSV tk gene [−50 to −57 **(G)**, −98 to −105 **(H)**; McKnight and Kingsbury, 1982]. Homologies depicted as bars above the ADA sequence are in the same strand, those drawn below the sequence are present in the opposite strand of the homologous promoter. Numbers indicate the corresponding positions in their own promoters, when known (authors' numbering). Arrows indicate the 16-bp direct repeat elements; GGGCGGG boxes are also indicated.

(1984), who have cloned an ADA cDNA from normal T lymphoblasts in which 76 bp of additional sequence were found in comparison with other published cDNA sequences. This insertion was at position 678 (authors' numbering), and we can now confirm their claim that this insert represents an intron as we have found this sequence in the genome between exons 7 and 8. Furthermore, Adrian *et al.* (1984) describe an ADA-deficient cell line (GM2294) in which the ADA mRNA transcribed from at least one allele contains a deletion of ~70 bp just 5′ to the splice site for the aforementioned 76-bp intron. Since we have found a small exon of 72 bp at this position in the genome, we suggest that due to aberrant splicing in this cell line, the sequence of exon 7 is absent in the mature ADA messenger described by Adrian *et al.*

Materials and methods

Isolation and characterization of genomic clones

The cosmid library was kindly provided by Dr. J. H. J. Hoeijmakers (Erasmus University, Rotterdam). It had been constructed from an *Mbo*I partial digest of human placental DNA, cloned into the *Bam*HI site of the cosmid vector pTCF (Grosveld *et al.*, 1982). Colony hybridization of this library was performed as described (Maniatis *et al.*, 1982). For the construction of subclones, restriction fragments were isolated from low melting agarose gels and ligated into convenient sites of plasmids pUC12 and pUC13 (Vieira and Messing, 1982). For DNA sequencing, DNA fragments were either sequenced directly by the procedure of Maxam and Gilbert (1980) or cloned into phage vectors M13mp8 and M13mp9 (Messing and Vieira, 1982) and sequenced by primed DNA synthesis with dideoxy chain terminators (Sanger *et al.*, 1977). All other DNA manipulations, including construction of the ADA minigenes, were performed according to established techniques (Maniatis *et al.*, 1982).

Analysis of the 5′ end of the ADA mRNA

RNA isolation, primer extension and S1 nuclease analysis were carried out according to Maniatis *et al.* (1982). In order to obtain an internally labeled S1 probe with high specific activity (~2 x 10^8 c.p.m./μg DNA), a DNA fragment that runs from the 5′ *Eco*RI site of pPRE to a *Taq*I site located 21 bp 3′ of the *Nco*I site, was cloned into M13mp8 (*Eco*RI/*Acc*I). The single-stranded form of this clone (0.1 μg) was hybridized to the M13 sequencing primer (a generous gift of Dr. J. Maat, Unilever Research Laboratory) and used as a template for elongation by Klenow's polymerase. The reaction conditions were: 6 mM Tris-HCl (pH 7.5), 50 mM NaCl, 6 mM $MgCl_2$, 6 mM DTE, 0.35 mM dATP and TTP, 4.5 μM dGTP and dCTP, 20 μCi [α-^{32}P]-dGTP and -dCTP (Radiochemical Centre, Amersham), and 10 units of Klenow's polymerase (Boehringer, Mannheim). The incubation was for 90 min at room temperature. The extended product was cut with *Nco*I and *Fnu*DII, and fractionated on a 5% polyacrylamide gel, from which the 103-bp fragment was isolated. This fragment was subsequently strand-separated. The single-stranded ^{32}P-labeled DNA fragment was hybridized to 2.5 μg of poly(A)-selected MOLT-4 RNA, digested with 1000 units of S1 nuclease (Sigma) per ml at 37°C and separated on a 7 M urea/8% polyacrylamide gel.

Transfer of the ADA minigene into mouse cells and assay for ADA activity

DNA transfections were performed as described by Lopata *et al.* (1984). Mouse L cells, routinely grown in Minimal Essential Medium (MEM) (without heavy metals) supplemented with 10% inactivated newborn calf serum (NCS), were seeded into 60 mm dishes at 5 x 10^5 cells/dish. 24 h later, 15 μg/ml of plasmid DNA and 200 μg/ml of DEAE dextran (Pharmacia) was added to the medium. After 20 h, the transfection solution was removed and replaced with 1 ml of HBS (137 mM NaCl, 5 mM KCl, 0.7 mM Na_2HPO_4, 6 mM dextrose and 21 mM Hepes, pH 7.05) containing 10% DMSO for 20 min at room temperature. After 48 h of further cultivation in MEM with 10% NCS, the cells were harvested and lysates prepared. These were assayed for the presence of human ADA activity by cellulose acetate gel electrophoresis followed by *in-situ* staining for ADA activity as described previously (Meera Khan, 1971).

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