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The β_2-microglobulin mRNA in human Daudi cells has a mutated initiation codon but is still inducible by interferon

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The human Burkitt lymphoma cell line Daudi does not synthesize β_2-microglobulin (β_2m) and lacks the cell surface histocompatibility antigens. The cells, however, contain RNA hybridizing to a cloned human β_2m cDNA probe. cDNA from this Daudi β_2m RNA, was cloned and sequenced. By comparison with cDNA prepared from Ramos cells, which synthesized microglobulin, we determined the sequence of the 20 amino acid long leader peptide of pre-β_2m and show that in Daudi cells the initiator ATG has been mutated to ATC. Although Daudi β_2m RNA cannot be translated, interferon induces the β_2m RNA in Daudi cells as well as in normal human cells.

Key words: interferon/initiation codon mutation/β_2-microglobulin/Daudi cell

Introduction

The Daudi cell line established from a Burkitt lymphoma (Klein *et al.*, 1968) does not synthesize detectable amounts of β_2-microglobulin (β_2m) (Nilsson *et al.*, 1974; Ploegh *et al.*, 1979; Ziegler *et al.*, 1981). Somatic cell genetic studies (Arce-Gomez *et al.*, 1978; Fellous *et al.*, 1977) have suggested that the lack of β_2m might be responsible for the absence of expression of HLA-A, B, C antigens in these cells. Ploegh *et al.* (1979) showed that RNA from Daudi cells directs the synthesis of the HLA-A, B, C heavy chains as efficiently as the RNA from other human lymphoblastoid cells. We recently showed that treatment with highly purified interferons (IFNs) enhances the amount of HLA-A, B, C heavy chain mRNAs in human cells including the Daudi cell line (Fellous *et al.*, 1982). Similarly, IFN treatment increases the amounts of β_2m mRNA in human cells (Wallach *et al.*, 1982). While studying the effects of IFN on Daudi cells, we discovered that despite the lack of detectable β_2m protein synthesis in these cells, an RNA hybridizing to β_2m cDNA is present in Daudi cells and is induced by IFN (Rosa *et al.*, 1982). This contrasted with the inability of RNA from Daudi cells to direct the synthesis of β_2m in cell-free systems (Ploegh *et al.*, 1979) and suggested that the Daudi β_2m mRNA might be defective. By cloning and sequencing of the β_2m cDNA prepared from IFN-treated Daudi cells, we show here that the Daudi β_2m mRNA has a point mutation in its initiation codon. The structure of the 5′-untranslated and leader peptide sequences of β_2m mRNA from another Burkitt lymphoma-derived cell line, Ramos, was also determined for comparison.

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Results

Cloning in Escherichia coli of β_2m cDNA prepared from Daudi cells

To detect β_2m mRNA, we used a cloned β_2m cDNA clone isolated in an *E. coli* plasmid by Suggs *et al.* (1981) from the RNA of the Burkitt lymphoma-derived Raji cell line. When hybridized to electrophoretic nitrocellulose blots of poly(A)$^+$ RNA from another Burkitt cell line, Ramos, the [^{32}P]β_2m cDNA probe detects a broad RNA band migrating as a 0.9–1.0 kb population (Rosa *et al.*, 1982). By the same analysis, Daudi cells were found to contain an RNA with almost similar electrophoretic mobility hybridizing to the β_2m cDNA probe. To investigate the structure of this Daudi RNA we prepared a library of cDNA clones.

Poly(A)$^+$ RNA from IFN-treated Daudi cells was prepared by the LiCl method (Auffray and Rougeon, 1980) and fractionated on a sucrose gradient. Fractions of RNA hybridizing to the Raji β_2m cDNA were reverse transcribed and converted to double-stranded cDNA by avian myeloblastosis virus reverse transcriptase. They were then treated with S1 nuclease, elongated by dCTP with terminal transferase and annealed to pBR322 cut with *Pst*I and tailed with dG. *E. coli* 1024 was used to clone recombinant plasmids and the tetracycline-resistant, ampicillin-sensitive colonies were hybridized *in situ* with the nick-translated, [^{32}P]β_2m cDNA probes described in Materials and methods. Six positive clones were selected for further analysis and clone 1 was finally sequenced by the procedures of Maxam and Gilbert (1980) as outlined in Figure 1.

Nucleotide sequence of the β_2m cDNA clone from Daudi cells

The sequence of Daudi β_2m cDNA clone 1 is compared in Figure 2 to that of the Raji β_2m cDNA clone of Suggs *et al.* (1981). The similarity of the sequences showed clearly that the Daudi clone is indeed derived from a β_2m mRNA. The Daudi cDNA spanned the coding region of β_2m mRNA with the exception of the last 22 codons, but was 42 nucleotides longer at its 5′ end than the Raji β_2m cDNA clone. Only three nucleotide differences were found between Daudi and Raji cDNAs, all three being silent replacements in the third base of a codon. Since the Raji β_2m cDNA clone of Suggs *et al.* (1981) is clearly incomplete at its 5′ end, we searched for the initiator ATG codon in the 42 extra nucleotides in the Daudi β_2m cDNA, but were unable to find it. Comparison with the sequence of the mouse pre-β_2m leader peptide determined by protein sequencing (Lingappa *et al.*, 1979) and more recently by gene sequencing (Parnes and Seidman, 1982) suggested, however, that the initiator methionine codon should be nine codons before the start of the Raji β_2m cDNA sequence of Suggs *et al.* (1981). Since at this position the Daudi β_2m cDNA had an ATC codon, we suspected that, in Daudi cells, the initiator ATG of the β_2m mRNA might have been mutated to ATC.

To verify this, we determined the 5′-terminal sequence of β_2m cDNA prepared from the Burkitt lymphoma-derived Ramos cells, which synthesize the β_2m protein normally (Fellous *et al.*, 1981) and compared it directly to that of the

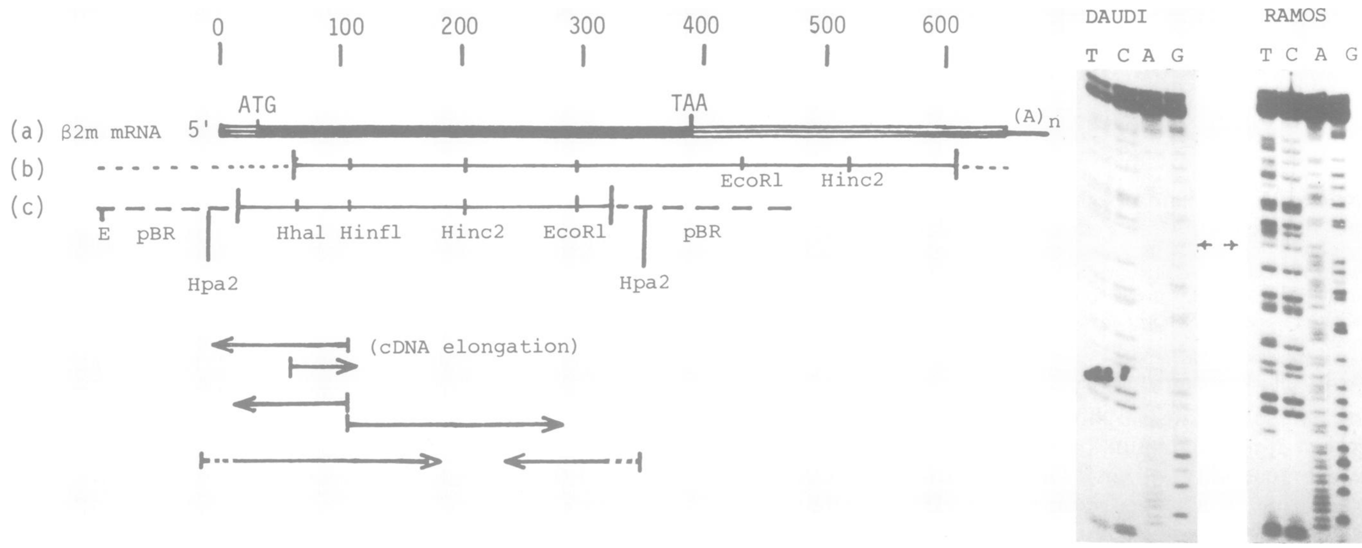

Fig. 1. β_2m mRNA and cDNA clones. The structure of human β_2m (**a**), is deduced from the sequence of the Raji β_2m cDNA clone of Suggs *et al.* (1981) shown in (**b**), and the sequence of the Daudi β_2m clone 1 (**c**). The length of the 3′-untranslated region near the poly(A) tail is only estimated by analogy to the mouse β_2m gene (Parnes and Seidman, 1982). The arrows show the sequencing strategy used for Daudi β_2m cDNA clone 1. The cDNA elongation experiment with the *Hinf*I-*Hha*I primer, is shown on the right (cDNA synthesis was carried out on total poly(A)$^+$ RNA from Daudi or Ramos cells with the 5′ end-labelled *Hinf*I-*Hha*I anti-sense primer derived from β_2m cDNA). The mutated nucleotide in Daudi is shown by arrows. The sequence is given in Figure 3. Note: the gels show the anti-sense strand, 5′ at the top.

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                 10        20        30        40        50        60        70        80        90

Daudi   1                                                        ...AGCATTCGGGCCGAGATCTCTCGCTCCGTGGCCTTAGCTGTG
                                                                                   s  r  s  v  a  l  a  v
            Hhal                                  Hinfl
Daudi  43   CTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCAGCGTACTCCAAAGATTCAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCC
Raji    1   CTCGCGCTACTCTCTCTTTCTGGCCTTGAGGCTATCCAGCGTACTCCAAAGATTCAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCAAATTTCC
             l  a  l  l  s  l  s  g  l  e  a  I  Q  R  T  P  K  I  Q  V  Y  S  R  H  P  A  E  N  G  K  S  N  F
                                               Hinc2
Daudi 143   TGAATTGCTATGTGTCTGGGTTTCATCCATCCGACATTGAAGTTGACTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGAGCATTCAGACTTGTCTTT
Raji  101   TGAATTGCTATGTGTCTGGGTTTCATCCATCCGACATTGAAGTTGACTTACTGAAGAATGGAGAGAGAATTGAAAAAGTGGAGCATTCAGACTTGTCTTT
            L  N  C  Y  V  S  G  F  H  P  S  D  I  E  V  D  L  L  K  N  G  E  R  I  E  K  V  E  H  S  D  L  S  F
                                          EcoR1
Daudi 243   CAGCAAGGACTGGTCTTTCTATCTCTTGTACTACACTGAATTCACCCCCACTGAAAAAGATGAGT
Raji  201   CAGCAAGGACTGGTCTTTCTATCTCTTGTATTATACTGAATTCACCCCCACTGAAAAAGATGAGTATGCCTGCCGTGTGAACCACGTGACTTTGTCACAG
              S  K  D  W  S  F  Y  L  L  Y  Y  T  E  F  T  P  T  E  K  D  E  Y  A  C  R  V  N  H  V  T  L  S  Q

Raji  301   CCCAAGATAGTTAAGTGGGATCGAGACATGTAAGCAGCATCATGGAGGTTGAAGATGCCGCATTTGGATTGGATGAATTCAAAATTCTGCTTGCTTGCTT
             P  K  I  V  K  W  D  R  D  M

Raji  401   TTTAATATTGATATGCTTATACACTTACACTTTATGCACAAAATGTAGGGTTATAATAATGTTAACATGGACATGATCTTCTTTATAATTCTACTTTGAG

Raji  501   TGCTGTCTCCATGTTTGATGTATCTGAGCAGGTTGCTCCACAGGTAGCT...
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Fig. 2. Nucleotide sequences of β_2m cDNA clones. The sequence of the inserts of the β_2m cDNA clone 1 from Daudi cells is compared to that of the clone from Raji cells (Suggs *et al.*, 1981) shown in Figure 1. The nucleotide differences and the mutated initiator codon are underlined. The cDNA clones are incomplete on the 5′ end (see Figure 3) and on the 3′ end (see Parnes and Seidman, 1982).

Daudi β_2m cDNA. Poly(A)$^+$ RNA from these two cell lines was used as template to synthesize cDNA using as primer the 50-nucleotide long *Hha*I-*Hinf*I fragment of β_2m cDNA clone 1 shown in Figure 1. The anti-sense strand of the primer was ^{32}P-labeled at the 5′-protruding end of the *Hinf*I site, and after elongation this strand was sequenced as shown in Figure 1 (right). Comparison of the Ramos and Daudi sequences obtained showed clearly that Ramos has the sequence 3′-TAC-5′ (sense: 5′-ATG-3′) where Daudi has 3′-TAG-5′ (sense: 5′-ATC-3′). No other difference was seen between the two sequences which include the 5′-untranslated region and the codons of the leader peptide of pre-β_2m (Figure 3).

The mutation of the first ATG codon to ATC in the Daudi β_2m mRNA sequence would be sufficient to explain the absence of β_2m protein synthesis in these cells. No other ATG codon in the sequence could serve to initiate the β_2m protein or other polypeptides of significant size (Figure 2). Since the reverse transcription reaction of total poly(A)$^+$ RNA from Daudi cells, initiated by the β_2m primer fragment, gave a unique sequence showing the mutation ATC codon (Figure 1, right) we can conclude that all the β_2m polyadenylated transcripts of Daudi cells have this mutation.

The RNA used for the reverse transcription reaction was extracted from Daudi cells which had been treated with 1000 U/ml purified IFN-α. Treatment with IFN-α or IFN-β produced a 3- to 4-fold increase in the amount of β_2m mRNA in these cells (Rosa *et al.*, 1982). The fact that only the mutated sequence was found in the cDNA elongation experiment of Figure 1 (right panel) shows that the treatment with IFN induces the defective β_2m mRNA in Daudi cells.

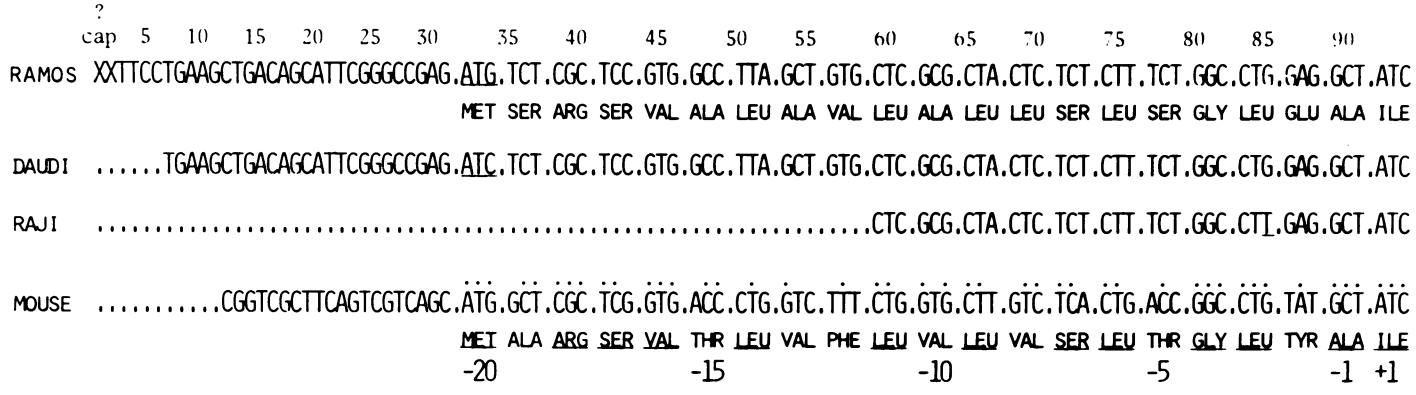

Fig. 3. 5′ End of the β_2m mRNA from human and mouse cells. The human sequence was determined from Ramos and Daudi by cDNA elongation as shown in Figure 1. The Raji sequence is from Suggs *et al.* (1981) and the mouse sequence from Parnes and Seidman (1982). The first amino acid of the mature β_2m protein is isoleucine (see Gates *et al.*, 1981). The mutated codon in Daudi and the sequence analogies between mouse and human mRNAs are indicated (underlined amino acid and dotted nucleotides).

Sequence of the leader peptide of human pre-β_2m

The length of the cDNA sequence synthesized from the primer in the experiment shown in Figure 2 was the same for Ramos and Daudi RNAs and suggests that the 5′ end of the β_2m mRNA is 31 nucleotides upstream from the initiator ATG codon. This experiment also allowed us to determine the amino acid sequence of the leader peptide of human pre-β_2m (Figure 3). As in the mouse (Parnes and Seidman, 1982), it is 20 amino acids long and 12 amino acids are identical to the mouse sequence. As expected, most of the hydrophobic residues are conserved. The length of the leader found is in good agreement with the decrease in size of pre-β_2m processed by endoplasmic reticulum membranes (Ploegh *et al.*, 1979). The analogy between the mouse and human nucleotide sequence in the 5′-terminal part of the β_2m mRNA, is clear only in the coding region but absent from the 5′-untranslated segment (Figure 3). This finding also supports the conclusion that the first ATG codon of the sequence is the initiator codon, which is mutated in Daudi cells.

Discussion

The absence of β_2m synthesis in the Daudi cell line derived from a Burkitt lymphoma has been known for many years from *in vivo* and *in vitro* translation experiments (Nilsson *et al.*, 1974; Ploegh *et al.*, 1979). Only recently however, using the cloned human β_2m cDNA probe obtained from Raji cells by Suggs *et al.* (1981) was it established that Daudi cells contain 13S RNA that hybridizes to β_2m cDNA (Rosa *et al.*, 1982; Depreval and Mach, 1982). It remained, nevertheless, possible that this RNA was only cross-hybridizing to the β_2m cDNA probe. The isolation and sequencing of a β_2m cDNA clone from Daudi cells described here, demonstrates that these cells do contain β_2m mRNA sequences. The Daudi cDNA sequenced, covers 80% of the β_2m coding region determined for the Raji β_2m cDNA clone (Suggs *et al.*, 1981) and the 98.9% homology between the two nucleotide sequences in this segment clearly indicates the identity between the two mRNAs. Only three silent nucleotide replacements in the third base of a codon were found in the sequence of the Daudi clone compared with that of the Raji clone.

By reverse transcription of RNA using as primer a 50 nucleotide-long fragment of β_2m cDNA, we determined the sequence of the missing 5′ end of β_2m mRNA. We compared directly the sequence obtained for RNA from Ramos cells, which synthesize β_2m, to that of the inactive RNA from Daudi cells. The only nucleotide difference found in the first 100 nucleotides studied is a G to C transversion in the first ATG codon of the Daudi sequence. It is interesting to note that this change also affects the third base of a codon. Several strong arguments support the conclusion that this mutation is in the initiator methionine codon. First, this appears to be the first ATG codon of the β_2m mRNA sequence and eukaryotic initiation usually occurs at the first ATG (Kozak, 1978). Since the primer-initiated reverse trancriptions gave a product of the same length in both Ramos and Daudi reactions, it is likely that the enzyme reached the 5′ terminus of the β_2m mRNA. The presence of secondary structure which would stop the enzyme is made unlikely by the strong RNA denaturing treatment used before the reaction. A second argument is that this ATG codon would put the initiator methionine at 20 residues before the isoleucine residue which is the amino-terminal end of the mature β_2m protein (Cunningham *et al.*, 1973). The leader peptide of human pre-β_2m would then have the length predicted by *in vitro* processing experiments (Ploegh *et al.*, 1979), and be identical in size as well as extensively homologous to the sequence of the leader peptide of mouse pre-β_2m (Lingappa *et al.*, 1979; Parnes and Seidman, 1982).

The point mutation in the initiation codon of Daudi β_2m mRNA would be sufficient to explain the lack of synthesis of any β_2m-like protein both *in vivo* and in mRNA translation assays (Ploegh *et al.*, 1979; Depreval and Mach, 1982) and is consistent with the fact that only a unique ATG codon can serve as initiator in eukaryotic mRNAs (Lomedico and McAndrew, 1982). Experiments are in progress to determine whether, because of the initiation defect, Daudi β_2m mRNA is less efficiently bound to ribosomes when incubated in a cell-free reticulocyte system. The 3′ end of the Daudi β_2m mRNA was not studied and may also be different. The slightly slower electrophoretic mobility observed in formamide/formaldehyde-agarose gels (Rosa *et al.*, 1982) may actually reflect such a difference in the sequence, or more likely in the length of the poly(A) tail. An important finding is that all the β_2m mRNA of Daudi cells appears to have the mutated initiation codon, since no other sequence was found in the reverse transcription reaction. This suggests that only one of the allelic β_2m genes is active in these cells. Either there is allelic exclusion, or more probably this results from the known rearrangement in one of the two chromosomes 15 of

Daudi cells. Arce-Gomez *et al.* (1978) showed that Daudi-mouse A9 hybrid cells, which contain the apparently normal human chromosome 15 of Daudi cells, also do not express β_2m. It is possible that this chromosome carries the β_2m gene with the mutated initiation codon. We are analyzing the DNA of such hybrids by the Southern blotting technique. The situation in Daudi cells would then resemble that in a mouse mutant thymoma cell line unable to synthesize β_2m, in which both allelic β_2m genes were found to be abnormal (Parnes and Seidman, 1982). Although point mutations affecting the expression of mRNAs have been described in man, as in thalassemias for example (Weatherall and Clegg, 1976), the Daudi β_2m mRNA may be the first example of a mutation in the initiation codon of a human gene. The otherwise very conserved β_2m mRNA sequence suggests that the mutation is recent and genetic drift has not yet occurred; we do not know if the mutation occurred in the tumor cells or may have affected other tissues of the patient.

The present experiments were prompted by our interest in the effect of IFN on the accumulation of HLA and β_2m mRNAs (Fellous *et al.* 1982; Wallach *et al.*, 1982). IFN treatment produced a 3- to 4-fold increase in β_2m mRNA in the Daudi cells, similar to that observed in normal human lymphoblastoid cells (Rosa *et al.*, 1982). Since all the β_2m mRNA of Daudi cells lacks the initiation codon and is therefore untranslatable, we can conclude that the effect of IFN does not require initiation of translation of the β_2m mRNA. It is likely that, as proposed for the (2′ – 5′) oligo(A) synthetase mRNA (Shulman and Revel, 1980), the IFN effect is on the synthesis of β_2m mRNA. Daudi cells, which are very sensitive to the anti-cellular effects of IFN (Adams *et al.*, 1975), provide an example of gratuitous mRNA induction in eukaryotic cells.

Materials and methods

Cell cultures

The human cell lines Daudi (Klein *et al.*, 1968) and Ramos (Klein *et al.*, 1976) were derived from Burkitt lymphomas and carry μ and κ chain immunoglobulins on their surface. The cells were grown in RPMI 1640 with fetal calf serum at 37°C with 5% CO_2. The Daudi cells were checked for their known immunological and cytogenetic markers (Arce-Gomez *et al.*, 1978) such as absence of HLA-A, B, C or β_2m but presence of HLA-DR surface antigens, abnormal chromosome 14 and deletion q14q21 near the centromeric region of chromosome 15. When indicated, cultures were treated for 4–6 h with 1000 U/ml of IFN-α (sub-species β3, 26 000 mol. wt.) purified to electrophoretic homogeneity (Rubinstein *et al.*, 1981) or with IFN-β as before (Fellous *et al.*, 1982).

β_2m mRNA and cDNA cloning

Total cellular RNA was prepared by the LiCl method (Auffray and Rougeon, 1980; LeMeur *et al.*. 1981). About 2–4 x 10^8 lymphoblastoid cells were washed with serum-free medium and homogenized for 1 min in a Waring Blendor with 20 ml of 3 M LiCl/6 M urea/10 mM sodium acetate pH 5 with 200 μg/ml heparin and 0.1% SDS. After 48 h at 4°C the pellet was centrifuged at 15 000 *g* for 30 min, washed twice with 20 ml of 4 M LiCl/8 M urea and dissolved in 5 ml of 0.1 M sodium acetate, pH 5/0.1% SDS. The RNA was extracted with 2 vol phenol/chloroform (1:1), then with 1 vol chloroform/ isoamylalcohol (24:1) and ethanol precipitated. The RNA was dissolved in 0.5 M NaCl/10 mM Tris-HCl, pH 7.5/0.1% SDS and passed three times through an oligo(dT)-cellulose column (PL Biochemicals, type 7). After washing with 0.05 M NaCl in the same buffer, the poly(A)$^+$ RNA was eluted in 10 mM Tris-HCl, pH 7.5/0.05% SDS and ethanol precipitated. Electrophoresis in formaldehyde agarose gels, blotting to nitrocellulose (Thomas, 1980) and hybridization to [^{32}P]cDNA was carried out as detailed before (Fellous *et al.*, 1982).

A total of 100 μg of poly(A)$^+$ RNA from IFN-treated Daudi cells was fractionated by centrifugation on a 15–30% sucrose gradient and the RNA fractions hybridizing the β_2m cDNA were identified on electrophoretic blots. The β_2m mRNA enriched fractions (2 μg) were used to prepare double-stranded cDNA by usual methods (Rougeon and Mach, 1977; Aleström *et al.*, 1980). The first strand was synthesized with AMV reverse transcriptase (a gift from J. Beard) for 45 min at 42°C with oligo(dT) 14–18 and the addition of 4 mM sodium pyrophosphate (Myers and Spiegelman, 1978). After alkali digestion, the single-stranded cDNA labeled with [^{32}P]dCTP (yield 25%) was isolated on Sephadex G-75 and heated to 65°C. The second strand was synthesized similarly with AMV reverse transcriptase for 2 h at 42°C without pyrophosphate, the duplex digested by 5 U S1 nuclease for 30 min at 30°C (50% resistance) and purified by sucrose gradient centrifugation. After tailing with dCTP by terminal transferase for 4 min at 35°C, the enzyme was inactivated by 20 mM EDTA/0.2% SDS and the tailed cDNA was annealed to pBR322 which had been cut by *Pst*I and tailed with dGTP (Roskam and Rougeon, 1979). *E. coli* C600 (1024) was transformed by established procedures (Aleström *et al.*, 1980) and 1500 amp^s tet^R colonies were hybridized *in situ* (Grunstein and Hogness, 1975) to the insert of the β_2m cDNA clone of Suggs *et al.* (1981). The 600-bp insert of this plasmid was excised by *Pst*I, redigested by *Hpa*II to eliminate remaining pBR sequences, purified by electrophoresis on a 5% polyacrylamide gel and labeled with [^{32}P]dCTP and dTTP by nick-translation (Rigby *et al.*, 1977; Fellous *et al.*, 1982). Filters were pre-hybridized 3 h at 65°C in 6 x SSC (0.15 M NaCl/0.015 M sodium citrate pH 7) with 1 x Denhardt's solution (Denhardt, 1966), then hybridized for 16 h at 45°C with 10^6 c.p.m. β_2m cDNA in 2 x SSC/1 x Denhardt/2 mM EDTA/0.07% SDS/25 mM KH_2PO_4 pH 7.2, and washed four times for 1 h at 68°C in 2 x SSC/1 x Denhardt/0.5% SDS. After autoradiography, six positive colonies were identified. Clone 1 was used for sequencing.

Nucleotide sequencing of Daudi β_2m cDNA

Plasmid DNA was purified by CsCl/ethidium bromide banding from lysozyme/SDS lysates (Guerry *et al.*, 1973). The restriction enzyme sites *Hinf*I, *Hha*I and *Hpa*II, shown in Figure 1, were used for end labeling and sequencing by the methods of Maxam and Gilbert (1980). For primer-dependent cDNA synthesis, a primer was prepared by cutting Daudi β_2m clone 1 plasmid DNA (24 μg) with *Bgl*II and *Eco*RI, isolating the 260-bp fragment on a 5–20% sucrose gradient and recutting by *Hha*I and *Hinf*I. The resulting 50-bp fragment was treated by alkaline phosphatase and 5′-end labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP. After denaturation for 10 min at 80°C, the two strands were separated by electrophoresis on a 6% denaturing polyacrylamide gel as described by Maxam and Gilbert (1980). The slow-moving anti-sense strand, labeled at the 5′ end of the *Hinf*I site (1.6 x 10^5 c.p.m.) was eluted, mixed with 100 μg total poly(A)$^+$ RNA from either IFN-treated Daudi or Ramos cells, extracted once by phenol, twice by ether and precipitated by ethanol. After heating for 2 min at 100°C and 10 min at 65°C in 50 mM Tris-HCl pH 8.2/10 mM $MgCl_2$/75 mM KCl/10 mM dithiothreitol, AMV reverse transcriptase (40 U) and 5 mM of each dXTP were added and elongation was carried out for 2 h at 37°C. After extraction with phenol/chloroform and precipitation by ethanol, the cDNA was fractionated on a 6% polyacrylamide sequencing gel. The band in the region of 100–150 bp was eluted and sequenced (Maxam and Gilbert, 1980). The sequence gels were exposed for 9 days to Agfa Curix X-ray films at –20°C.

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