Proc. Natl. Acad. Sci. USA
Vol. 78, No. 4, pp. 2253–2257, April 1981
Biochemistry

Mouse β_2-microglobulin cDNA clones: A screening procedure for cDNA clones corresponding to rare mRNAs

(cDNA cloning/nucleotide sequence)

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Communicated by Philip Leder, January 12, 1981

ABSTRACT **We have isolated three cDNA clones for β_2-microglobulin, the small subunit of the major histocompatibility antigens. β_2-Microglobulin makes up less than 0.1% of mouse liver protein, and its mRNA is approximately 0.03% of liver poly(A)$^+$ mRNA. The cDNA clones were identified by screening 1400 cDNA clones made from 9–10S mouse liver poly(A)$^+$ mRNA. The procedure for screening the cDNA clones involved binding pooled plasmid DNA to nitrocellulose filters and testing the ability of each filter to select β_2-microglobulin mRNA. The filter-selected mRNAs were assayed for their ability to direct the synthesis of β_2-microglobulin in translation reactions *in vitro*. The isolated clones were shown by nucleotide sequence analysis to encode β_2-microglobulin. The positive-selection–hybridization assay has been modified to facilitate the screening of large numbers of cDNA clones, and the modified assay should allow the isolation of cDNAs corresponding to any mRNA whose *in vitro* translation products can be immunoprecipitated. These modifications are of particular value in the isolation of cDNA clones corresponding to rare species of mRNA.**

The application of recombinant DNA technology over the past several years has greatly increased our understanding of the arrangement of many eukaryotic genes. The use of this technology has required the synthesis of cDNA probes that are specific for particular genes. Insulin (1, 2), ovalbumin (3, 4), α and β globin (5–7), immunoglobulin light and heavy chains (8–12), and interferon (13) are just a few of the mRNA sequences that have been cloned in the form of cDNA. Almost all of these cloned mRNAs are found in differentiated cells, where they either represent a significant fraction of the total mRNA population or can be induced to a relatively high level. Nucleic acid hybridization, using the purified mRNAs or cDNAs as probes, was generally used to identify the correct cDNA clone. One exception to this procedure has been the cloning of interferon cDNA. The availability of a sensitive biological assay for interferon provided an alternative method for identifying the cloned cDNA (13). However, there are many mRNAs that are never expressed in large amounts and for which sensitive biological assays are not available. Procedures for cloning these rare mRNAs are currently being developed. Using one such procedure, Ploegh *et al.* (14) recently isolated a cDNA clone for HLA, the 45,000 M_r chain of a human major histocompatibility antigen.

β_2-Microglobulin is a short, nonglycosylated polypeptide (12,000 M_r) present in serum and, in small quantities, on the surface of virtually all mammalian cells. It makes up less than 0.1% of the total protein of most cells (unpublished results; ref. 15). No enzymatic activity has been associated with β_2-microglobulin, and in fact its function is not known. However, β_2-microglobulin is known to be one of the subunits of the major histocompatibility antigens and to be associated with at least two other cell surface glycoproteins encoded within the major histocompatibility complex. Until recently (16) the lack of recognized polymorphic forms of β_2-microglobulin limited classical genetic studies. Thus the organization of the β_2-microglobulin gene(s) has remained uncertain. As a first step toward understanding the arrangement of the gene(s) for β_2-microglobulin, we have cloned the β_2-microglobulin cDNA.

In order to clone β_2-microglobulin cDNA we have modified the procedure of positive-selection–translation so that large numbers of clones can be screened to isolate cDNA clones for proteins whose mRNA represents as little as 0.03% of the total cellular mRNA. Similar procedures have been proposed by others (17, 18); however, we believe that this report demonstrates the feasibility of this approach to cloning the cDNA of rare mRNA species. We describe here the screening procedure by which β_2-microglobulin cDNA clones were identified and the nucleotide sequence analysis of the isolated β_2-microglobulin cDNA clones.

MATERIALS AND METHODS

Construction of cDNA Clones. Total polysomal mRNA was extracted from livers of C57BL/6 mice according to the Mg^{2+} precipitation procedure of Efstratiadis and Kafatos (19). The poly(A)-enriched fraction was selected by oligo(dT)-cellulose chromatography and centrifuged through a linear sucrose gradient. The fractions were assayed for mRNA encoding β_2-microglobulin by *in vitro* translation and immunoprecipitation as described below. The fraction containing β_2-microglobulin mRNA (9–10S) was used for the synthesis of double-stranded cDNA according to the procedure of Villa-Komaroff *et al.* (2), which involves the use of poly(dG) and poly(dC) tails and insertion into the endonuclease *Pst* I site of plasmid pBR322; this allows the inserts to be excised by *Pst* I cleavage (2). Annealing and transformation of *Escherichia coli* strain LE392 were as described by Dagert and Ehrlich (20). Recombinant DNA was handled in accordance with the National Institutes of Health guidelines.

Clone Screening Procedure. Fig. 1 outlines the procedure used to screen cDNA clones. The individual steps are described below.

Preparation of plasmid DNA pools. Individual cDNA-containing clones were grown overnight at 37°C in 5 ml of tryptone broth containing tetracyline at 4 μg/ml, and then stored at 4°C. Pools of these isolates were made by inoculation of 0.3 ml of each of seven saturated cultures into 100 ml of brain heart infusion

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Abbreviation: bp, base pairs.

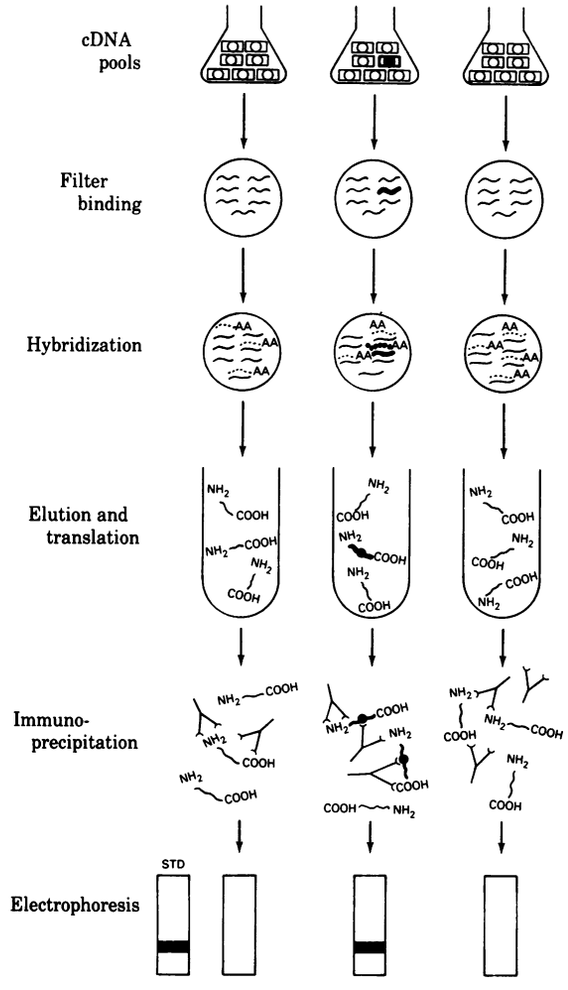

FIG. 1. Screening procedure used to identify β_2-microglobulin cDNA clones. The DNA from pools of plasmid-bearing strains was isolated, denatured, and bound to nitrocellulose filters. The filters were hybridized with mouse liver poly(A)$^+$ mRNA. Only a pool of clones containing a β_2-microglobulin cDNA clone (boldface, center pool) could hybridize to β_2-microglobulin mRNA (boldface). *In vitro* translation of this mRNA after elution, followed by immunoprecipitation with anti-β_2-microglobulin, gel electrophoresis, and autoradiography produced a band (center lane) that comigrated with the *in vitro* β_2-microglobulin standard. *Materials and Methods* contains a more detailed description of the procedures.

medium (Difco) containing tetracycline at 4 μg/ml and growth at 37°C until the OD_{590} reached 0.7. Chloramphenicol (100 μg/ml) was then added for plasmid induction, and cultures were incubated overnight at 37°C. DNA was isolated from the cultures through the cleared lysate as described (21), extracted with phenol, and precipitated with ethanol.

Filter binding. The ethanol-precipitated plasmid DNA was resuspended in 1.5 ml of 20 mM Tris·HCl, pH 7.6, containing 1 mM EDTA and boiled for 10 min. An equal volume of 1 M NaOH was added and samples were incubated at room temperature for 20 min. Samples were then neutralized by addition of 9 ml of a solution containing 1.5 M NaCl, 0.15 M sodium citrate, 0.25 M Tris·HCl at pH 8.0, and 0.25 M HCl. The DNA was immediately bound to nitrocellulose filters (Millipore, 2.5 cm diameter, 0.45-μm pores) by slow filtration ($\approx$1 ml/min) on a multifilter suction device (Yeda Scientific, Israel). Filters were washed extensively with 0.9 M NaCl/0.09 M sodium citrate and baked either overnight at 70°C or for 2 hr at 80°C in a vacuum oven. A sterile one-hole paper punch was used to obtain 0.5-cm discs from these filters for use in hybridization.

Hybridization and RNA elution. Filter hybridization and elution were performed by modification of the method of Ricciardi *et al.* (22). In a single silicone-treated glass vial, 40 μg of poly(A)$^+$ mouse liver polysomal RNA was heated to 70°C for 10 min in 0.5 ml of hybridization solution consisting of 65% (vol/vol) deionized formamide, 20 mM 1,4-piperazinediethanesulfonic acid (Pipes) at pH 6.4, 0.2% $NaDodSO_4$, 0.4 M NaCl, and yeast tRNA (GIBCO) at 0.1 mg/ml. Twenty 0.5-cm filters were then added and incubated at 50°C for 2 hr. The filters were transferred to a 50-ml plastic test tube and washed at 65°C by Vortex mixing and aspiration, nine times with 50 ml of 0.01 M Tris·HCl, pH 7.6/0.15 M NaCl/1 mM EDTA/0.5% $NaDodSO_4$ and two times with 50 ml of the same buffer without $NaDodSO_4$.

For elution of hybridized RNA the filters were transferred to silicone-treated Microfuge tubes, two per tube (yielding a pool of 14 clones in each tube); 300 μl of water and 30 μg of yeast tRNA were added. The tubes were placed in boiling water for 1 min, quick frozen in a dry ice/ethanol bath, and thawed at room temperature. The filters were removed and the eluted RNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (1:1:0.04, vol/vol). Sodium acetate, pH 5.0, was added to 0.2 M and the RNA was precipitated with 2 vol of ethanol. The precipitates were washed with 95% (vol/vol) ethanol, dried briefly under reduced pressure, and resuspended in 5 μl of water for translation *in vitro*.

Translation, immunoprecipitation, and gel electrophoresis. Each pool (14 clones) of eluted RNA was translated *in vitro* by using a rabbit reticulocyte lysate system (Bethesda Research Laboratories, Rockville, MD) and added [^{35}S]methionine (New England Nuclear). Immunoprecipitation of translation products with anti-β_2-microglobulin antiserum (23) and staphylococcal protein A-Sepharose (Pharmacia) was as described by Dobberstein *et al.* (24). Immunoprecipitated products were released from protein A-Sepharose beads by boiling 5 min in 25 μl of sample buffer (25) and electrophoresed in polyacrylamide/$NaDodSO_4$ gels (25). The gels were 17% polyacrylamide and the ratio of acrylamide to bisacrylamide was 300:1. Gels were fluorographed with dimethyl sulfoxide/2,5-diphenyloxazole (New England Nuclear), dried, and autoradiographed. Positive pools were identified by a band that comigrated with the *in vitro* β_2-microglobulin standard. As a control for the various steps in this screening procedure filter-bound pBR-K41(V+C) plasmid DNA containing immunoglobulin κ light chain sequences (8) was hybridized to MOPC-41 poly(A)$^+$ mRNA and the *in vitro* translation products were immunoprecipitated with rabbit antiserum to mouse κ light chain (Miles).

Characterization of Positive Clones. The procedures for determining the nucleotide sequences of the cDNA inserts have been described (2). First, restriction endonuclease sites within the inserts were identified. The fragments produced by cleavage with these enzymes were labeled by the action of polynucleotide kinase and the nucleotide sequences of the fragments were determined by the procedure of Maxam and Gilbert (26).

RESULTS AND DISCUSSION

Many proteins are never expressed at high levels, and thus their mRNAs are never made in large amounts in any particular cell. The genes encoding these proteins have so far not been amenable to study by recombinant DNA techniques because nucleic acid probes for these genes have not been available. One approach to making nucleic acid probes for these genes is to clone the cDNAs corresponding to the particular mRNA species. These rare mRNAs can be detected only by their ability to direct the synthesis of an *in vitro* translation product that can be immunoprecipitated by an antiserum and characterized by gel

electrophoresis. β_2-Microglobulin is one of these rare cellular proteins. Recently, Lingappa *et al.* (27) and Dobberstein *et al.* (24) showed that the poly(A)$^+$ mRNA from the murine tumor lines EL4 and SL2, respectively, could direct the synthesis of β_2-microglobulin in an *in vitro* translation reaction.

We have used *in vitro* translation to assay the purification of β_2-microglobulin mRNA (results not shown). Liver mRNA was fractionated on a sucrose gradient and the fraction containing β_2-microglobulin mRNA was identified by its ability to direct the synthesis of β_2-microglobulin. To confirm that the protein product immunoprecipitated by the anti-β_2-microglobulin serum was β_2-microglobulin, the product was labeled with [^{3}H]leucine and the locations of the leucine residues were determined in a Beckman model 890B amino acid sequencer. The five leucine residues of the *in vitro* product, at positions 6, 9, 11, 14, and 17 (unpublished results), corresponded exactly to the five leucine residues of the β_2-microglobulin signal peptide reported by Lingappa *et al.* (27). The β_2-microglobulin mRNA was found in the 9–10S fraction. This fraction represented about 10% of the total liver mRNA applied to the sucrose gradients, so there was a 10-fold purification of the β_2-microglobulin mRNA.

When the 9–10S mRNA was translated *in vitro*, less than 1% of the synthesized protein was immunoprecipitated (results not shown). Thus, the β_2-microglobulin mRNA made up less than 1% of the sucrose gradient-purified 9–10S mRNA. cDNA clones were made from this mRNA fraction by the procedures described by Villa-Komaroff *et al.* (2). From 5 μg of poly(A)$^+$ mRNA we obtained enough double-stranded cDNA to make more than 10,000 independent clones. More than 95% of these clones were sensitive to ampicillin and thus contained inserts.

We presumed that less than 1 cDNA clone in 100 would correspond to a β_2-microglobulin cDNA clone. Thus, we decided to screen these cDNA clones by the procedure described in Fig. 1. This procedure screens for the ability of a cDNA clone to hybridize to β_2-microglobulin mRNA; the mRNA is assayed by immunoprecipitation of the *in vitro* translation product. Approximately 1400 cDNA clones were screened by this method, and 4 positive pools of 14 were identified. The 42 plasmid strains from three of these positive pools were grown separately, and then plasmid DNAs were assayed for their ability to hybridize to β_2-microglobulin mRNA. Fig. 2 shows the β_2-microglobulin made from the pools containing the clones pBRcB-1 (lane c) and pBRcB-2 (lane a). Three nonsibling β_2-microglobulin clones were isolated by this procedure.

The three β_2-microglobulin cDNA clones were characterized by restriction endonuclease mapping and nucleotide sequence analysis (Figs. 3 and 4). The inserts [including poly(dG·dC) tails] in the clones pBRcB-1, pBRcB-2, and pBRcB-3 are 140, 310, and 265 base pairs (bp), respectively. The nucleotide sequence of pBRcB-1 clearly would encode murine β_2-microglobulin between amino acids 11 and 37 (Fig. 4 and refs. 28 and 29). There is a minor discrepancy between the two published amino acid sequences for murine β_2-microglobulin at position 29 [Gln (28) versus Glu (29)]. The nucleotide sequence we have determined predicts a Gln residue at this position. Clones pBRcB-2 and pBRcB-3 were found to contain overlapping sequences by comparison of restriction enzyme cleavage sites (Fig. 3) and nucleotide sequence (Fig. 4). These two clones contain sequences corresponding to approximately amino acid 50 through the carboxyl end of the protein (amino acid 99) and over 150 bp into the 3′ untranslated region. The portions of these clones whose sequences have been determined thus far are indicated in Figs. 3 and 4. The recently determined murine β_2-microglobulin amino acid sequence (28) and the amino acid sequence predicted from the nucleotide sequences of these cloned cDNAs

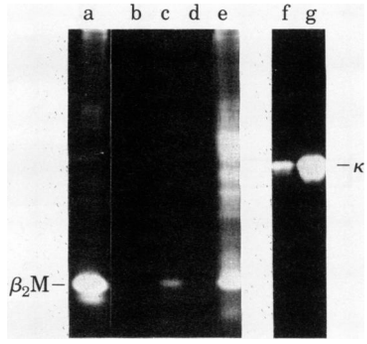

FIG. 2. Autoradiogram of a NaDodSO$_4$/polyacrylamide gel used to fractionate β_2-microglobulin immunoprecipitated from *in vitro* translation reactions. The immunoprecipitated products of *in vitro* translation reactions were programmed by mRNA selected by pools of plasmid DNA including clones pBRcB-2 (lane a) and pBRcB-1 (lane c) and by pools of clones lacking β_2-microglobulin sequence (lanes b and d). Mouse liver poly(A)$^+$ mRNA was translated and immunoprecipitated by β_2-microglobulin antiserum (lane e); the position of β_2-microglobulin (β_2M) is indicated. The autoradiogram is overexposed to display fainter bands corresponding to other proteins precipitated by the β_2-microglobulin antiserum. κ light chain was immunoprecipitated from an *in vitro* translation reaction programmed by 0.5 μg of MOPC-41 poly(A)$^+$ mRNA (lane g) and by the mRNA selected by a MOPC-41 κ light chain cDNA clone from 5 μg of MOPC-41 poly(A)$^+$ mRNA (lane f). The position of the κ light chain is indicated. The procedures for filter selection, translation, and immunoprecipitation were the same as those described for β_2-microglobulin except that the MOPC-41 cDNA clone, MOPC-41 poly(A)$^+$ mRNA, and rabbit antiserum to mouse κ chain were used.

are in perfect agreement. Thus, the cloned β_2-microglobulin cDNA sequences could encode the entire β_2-microglobulin mRNA with the exception of the 5′ and part of the 3′ untranslated regions and the regions encoding the signal peptide, amino acids 1–10, and amino acids 42–49.

Screening 1400 cDNA clones for β_2-microglobulin sequences yielded at least 3, and probably 4, β_2-microglobulin cDNA clones (the fourth clone has not been analyzed by nucleotide sequence determination). A screening procedure can be defective in two ways, either yielding false positive clones or allowing clones containing the correct sequence to escape detection. The screening procedure outlined in Fig. 1 yielded no false positives. That is, all of the clones that were selected on the basis of hybridization did indeed contain β_2-microglobulin cDNA sequences. However, the β_2-microglobulin antiserum does precipitate several products from a cell-free translation of poly(A)$^+$ liver mRNA (an overexposure of the precipitated products is displayed in Fig. 2, lane e). The cDNA clones corresponding to several of these other products were identified on the basis of this screening procedure. These clones were discarded because the protein product associated with them did not comigrate with β_2-microglobulin on the NaDodSO$_4$/polyacrylamide gel. Although we cannot be certain that no β_2-microglobulin cDNA clones escaped detection, we believe this to be the case. A clone might be expected to escape detection by this procedure if the inserted sequence is very short. However, we were able to detect a cDNA clone, pBRcB-1, containing as few as 100 bp of β_2-microglobulin coding sequence, although the amount of mRNA that hybridized to that clone was less than the amount of mRNA that hybridized to the longer clone, pBRcB-2 (Fig. 2, lanes c and a).

The number of cDNA clones that can be screened simultaneously by the procedure outlined in Fig. 1 is limited by the sensitivity of the translation–immunoprecipitation–NaDodSO$_4$/polyacrylamide gel system, because the latter determines the

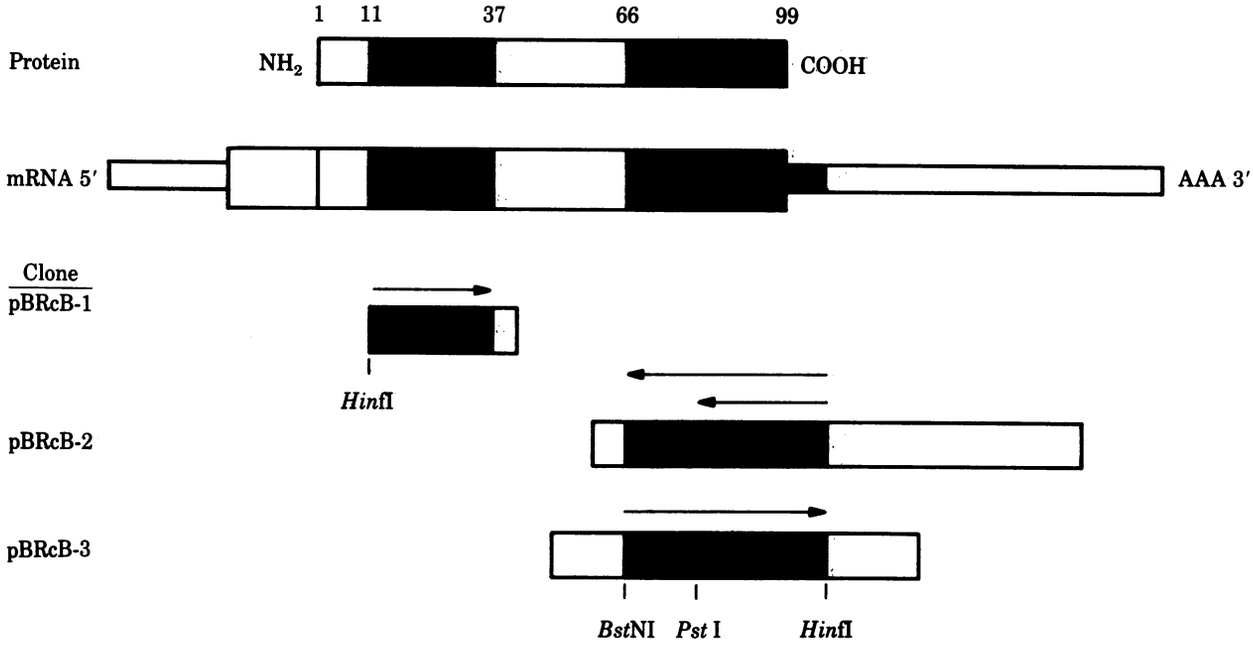

FIG. 3. Strategy for determining the nucleotide sequence of the inserts in the three β_2-microglobulin cDNA clones, pBRcB-1, pBRcB-2, and pBRcB-3. Maps of the β_2-microglobulin protein, mRNA, and cDNA clones were shown with black shading to indicate the regions whose sequences have been determined to date. Sequence analysis on each of the plasmids was done by isolating a *Cfo* I fragment that contained the entire insert plus 337 bp of plasmid sequence. This fragment was then cut by a second enzyme (*Hin*fI for pBRcB-1 and pBRcB-2 and *Bst*NI for pBRcB-3), dephosphorylated with bacterial alkaline phosphatase, and labeled with ^{32}P by the action of polynucleotide kinase. The labeled fragments were digested once more (*Pst* I for pBRcB-1, *Pst* I or *Bst*NI for pBRcB-2, and *Hin*fI for pBRcB-3) to yield fragments labeled at a single end for nucleotide sequence analysis. The nucleotide sequences determined from each of these fragments are indicated in Fig. 4. Arrows indicate direction and extent of sequence determination.

length of autoradiography exposure time necessary to detect a protein band. The more sensitive this detection system, the more cDNA clones can be bound to a single nitrocellulose filter. In several control experiments (similar to those shown in Fig. 2, lanes f and g) we found that the amount of mRNA selected by filter-bound immunoglobulin κ light chain plasmid DNAs decreased to below the detectable limit if less than 10 ml of culture was used to make the plasmid DNA (data not shown). Thus, a 100-ml culture could contain no more than 7–10 plasmid strains. Cultures larger than 100 ml could not be used because the DNA from larger cultures exceeded the capacity of a 2.5-cm (diameter) filter.

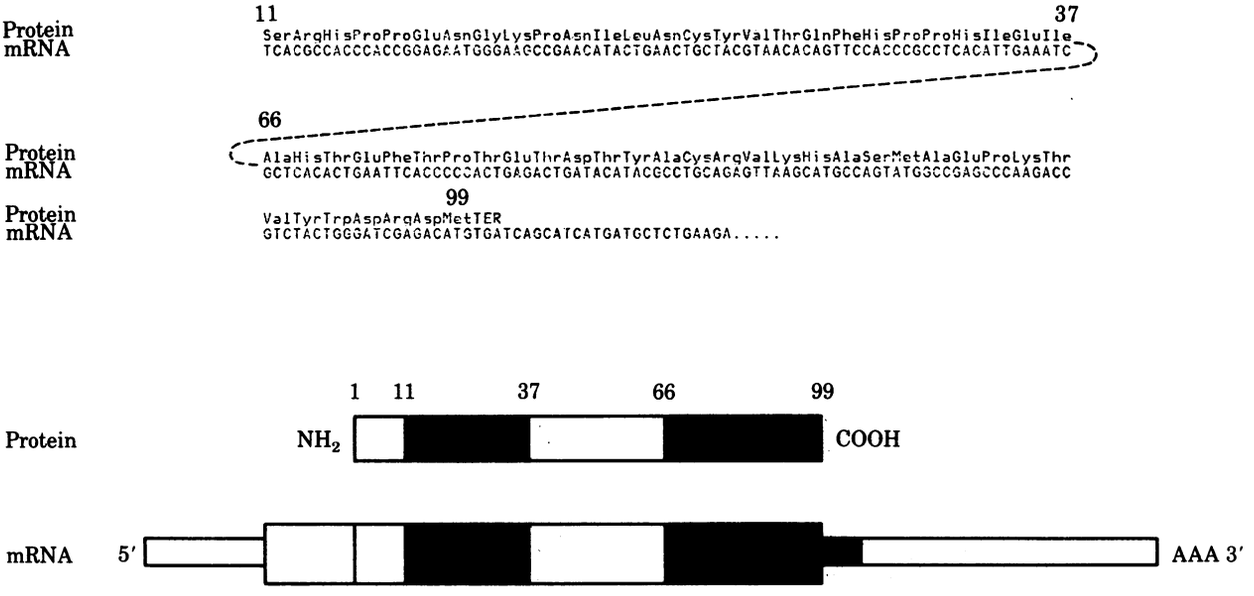

FIG. 4. Nucleotide sequences of the coding strands of the mouse β_2-microglobulin cDNA clones ("mRNA") and their translation into amino acid sequence ("protein"). (TER, termination.) The strategy for determining the nucleotide sequences is indicated in Fig. 3. The portions of the protein and mRNA that correspond to portions of the sequenced clones are shaded in the maps below. The nucleotide sequence predicts an amino acid sequence which corresponds to that of murine β_2-microglobulin at every position (28).

In order to obtain enough mRNA to detect the protein product by translation and immunoprecipitation, the mRNA added to the hybridization must be sufficient to saturate a filter to which plasmid from about 10 ml of culture has been bound. Of course one cannot know before a cDNA clone is isolated exactly how much total poly(A)$^+$ mRNA is required to saturate a plasmid filter. However, one can use the translation–immunoprecipitation system as an assay for the amount of specific mRNA present in the poly(A)$^+$ mRNA. For example, there is about 20- to 50-fold more κ light chain mRNA in plasmacytoma poly(A)$^+$ mRNA than there is β_2-microglobulin mRNA in liver poly(A)$^+$ mRNA (unpublished results). Thus, one requires 20- to 50-fold more liver mRNA than plasmacytoma mRNA to detect β_2-microglobulin rather than κ light chain cDNAs. [We assumed that the elution of mRNA from the filters would be the same for both mRNAs—approximately 10–30% (Fig. 2, lanes f and g).]

Various procedures have been used for isolating other cDNA clones. Most cDNA clones have been identified by hybridization to highly purified preparations of either mRNA or cDNA. The identity of cDNA clones isolated by this procedure was then confirmed by positive selection of the mRNA by using plasmid DNA or by nucleotide sequence of the plasmid DNA. The technique described here for the isolation of the β_2-microglobulin cDNA clones has involved modifications of the positive selection procedure. These modifications allow the screening of hundreds of cDNA clones with the same effort that was previously required to confirm the identity of a few selected cDNA clones. A few cDNA clones have been detected by using sensitive radioimmunoassays for the protein products produced in *E. coli* (30, 31). The procedure described here for the isolation of β_2-microglobulin cDNA clones has several advantages over the radioimmunological detection techniques. First, the radioimmunological screening procedure requires a sensitive solid-phase radioimmunoassay to detect the product of interest. These sensitive radioimmunoassays are frequently not available for the protein of interest. Second, this procedure requires that the portion of the mRNA encoding the antigenic site must actually be encoded by the cDNA clone. If the mRNA is a long one the frequency of cDNA clones containing these segments may be very low. Third, one cannot know if the radioimmunoassay will be able to detect fragments of the polypeptide attached to an *E. coli* protein. A major advantage of the procedure described for the cloning of β_2-microglobulin is that if one can detect the mRNA in an *in vitro* translation reaction then one can be certain that the cDNA clone will be detected by the screening procedure.

The β_2-microglobulin clones isolated by this procedure provide some information about β_2-microglobulin mRNA. For example, we can calculate that β_2-microglobulin mRNA is probably about 0.03% of the total liver poly(A)$^+$ mRNA, based upon the frequency of positive cDNA clones (4 in 1400) and the sucrose gradient purification (10-fold) of the poly(A)$^+$ RNA. We can also conclude that the 3′ untranslated region of the mRNA is more than 150 bases long. More importantly, these clones provide nucleic acid probes to examine the structure of the β_2-microglobulin gene(s). In the mouse, β_2-microglobulin has been identified as a subunit of at least five different proteins: H-2K, H-2D, TL, Qa-2, and a serum protein (32). While H-2K and H-2D are found on nearly every cell, TL is found only on lymphocytes from leukemic mice and thymocytes, and Qa-2 is found on lymphocytes (33). From the available data one cannot determine whether the β_2-microglobulin associated with these different proteins is encoded by the same gene or closely related but different genes. However, preliminary characterization of mouse β_2-microglobulin gene sequences by Southern blot analysis (34) suggests that there is only a single complete β_2-microglobulin gene. The gene has at least three intervening sequences (unpublished results). Eventually, we expect that these cloned cDNA probes will provide invaluable tools for the further analysis of the β_2-microglobulin gene.

We thank Terri Broderick for her help in preparing the manuscript. J.R.P. is the recipient of a National Research Service Award (1F32 AI05932-01) from the National Institute of Allergy and Infectious Diseases.

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