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# Isolation and partial nucleotide sequence of a cDNA clone for human histocompatibility antigen HLA-B by use of an oligodeoxynucleotide primer

(dideoxynucleoside triphosphate-terminated cDNA synthesis)

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**ABSTRACT** **We have isolated a cDNA clone for one of the HLA-B locus alloantigens by hybridization with a 30-nucleotide-long DNA probe. The probe was isolated from a reverse transcriptase (RNA-dependent DNA nucleotidyltransferase)-catalyzed cDNA synthesis reaction on poly(A)-mRNA in which an oligonucleotide (5′-$^{32}$P)dC-T-T-C-T-C-C-A-C-A-$T_{OH}$ served as a primer and in which dideoxynucleoside triphosphates were used to reduce the size and heterogeneity of the cDNA products. The desired cDNA clone was isolated from a library of recombinant cDNA clones in the plasmid pBR322. The partial nucleotide sequence of the cDNA clone corresponds to the amino acid sequence of HLA-B7 antigen. The approach described in this paper is extremely sensitive and may be useful in cloning other genes for which the corresponding mRNA is present at low levels. This cDNA clone is nearly full length and can be used to isolate and to study the genes within the HLA region and to obtain expression of HLA-B peptides in cells.**

Our understanding of the regulation of higher eukaryotic gene expression is intimately linked to our knowledge of the structure and organization of genes and their products. Several groups have directed their efforts in this direction with considerable success. These studies have mainly focused on those genetic loci that encode the highly predominant tissue-specific protein products [for example, globins, immunoglobulins, and ovalbumin (1–7)] or on genes for dihydrofolate reductase that may be amplified selectively in some cells (8).

With a view to studying the genes that are expressed in many cell types at lower levels, we became interested in the major histocompatibility complex in man called HLA. Included within this region are the genes encoding the serologically detected antigens (HLA-A, HLA-B, HLA-C); genes regulating immune response and other loci, including HLA-D; the structural genes for $C_2$ and $C_4$ components of the complement system and factor B of the alternative pathway; and genes for a blood group factor (9). The genes coding for HLA-A, HLA-B, and HLA-C antigens are expressed together in many cell types, and there is a quantitative difference in the levels of the HLA-A, HLA-B, and HLA-C antigens among various tissues (10). Furthermore, the antigens coded for by genes of HLA-A, HLA-B, HLA-C, and HLA-D loci exhibit an unusually high degree of genetic polymorphism. It is of considerable interest to study the mechanism regulating expression of these genes in various cell types and also to understand the molecular basis of the polymorphism exhibited by these antigens. The feasibility of the above study depends on the availability of the histocompatibility cDNA clone, which could be used as a specific probe to isolate the genes and to study the mRNAs and their precursors. This paper describes the isolation of a cDNA clone corresponding to the HLA-B locus antigens.

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## MATERIALS AND METHODS

The sources of chemicals for oligonucleotide synthesis were as described (11). Deoxynucleoside triphosphates, dideoxynucleoside triphosphates, and oligo(dT)-cellulose were purchased from Collaborative Research, Waltham, MA. Reverse transcriptase (RNA-dependent DNA nucleotidyltransferase) from avian myeloblastosis virus was supplied by J. W. Beard (Life Sciences, St. Petersburg, FL). [$\gamma$-$^{32}$P]ATP (specific activity, 3000 Ci/mmol; 1 Ci = $3.7 \times 10^{10}$ becquerels) was purchased from New England Nuclear.

Polynucleotide kinase was prepared as described (12). Human lymphoblastoid cell line RPMI 4265 (HLA-A2, B7, B12) was a gift from C. Terhorst.

**Preparation of Poly(A)-mRNA.** RPMI 4265 cells were grown to a density of $4 \times 10^5$ cells per ml in RPMI 1640 media containing fetal calf serum, glutamate, pyruvate, penicillin, and streptomycin. RNA was extracted as described by Ghosh *et al.* (13), except that phenol/chloroform/isoamylalcohol, 100:100:1 (vol/vol), was used in place of phenol extraction. Poly(A)-mRNA was isolated on oligo(dT)-cellulose column.

**Synthesis and Characterization of Oligonucleotide Primer.** The primer was chemically synthesized by the triester method (14, 15). It was phosphorylated at the 5′ end with [$\gamma$-$^{32}$P]ATP and T4 polynucleotide kinase to a specific activity of 1000–1500 Ci/mmol. The primer was characterized by the two-dimensional electrophoresis/homochromatography technique (16).

**Prehybridization of Primer/RNA Followed by cDNA Synthesis.** $^{32}$P-Labeled primer [0.25 $\mu$g (75 pmol)] was mixed with 80 $\mu$g of poly(A)-RNA in 60 $\mu$l of 80 mM KCl. The mixture was heated to 90°C for 10 min, followed by addition of 5 $\mu$l of 1 M Tris·HCl (pH 8.3). This mixture was incubated for 2 hr at 41°C. The dideoxynucleoside triphosphate-terminated cDNA synthesis reaction mixture (100 $\mu$l) contained the above primer/RNA mixture (60 $\mu$l), 5 mM $MgCl_2$, 10 mM dithiothreitol, 500 $\mu$M dideoxynucleoside triphosphate, 500 $\mu$M each of the other three deoxynucleoside triphosphates, and 5 $\mu$l of reverse transcriptase (30 units/$\mu$l). The reaction was incubated at 41°C for 3 hr; 125 $\mu$l of water was added, followed by extraction with 100 $\mu$l of phenol/chloroform, 1:1 (vol/vol). The aqueous phase was washed with either solvent made 0.3 M in NaOAc and treated with 3 vol of 95% ethanol. The precipitate was dissolved in 25 $\mu$l of 0.1 M NaOH and incubated at 41°C for 1 hr. An equal volume of 8 M urea/0.05% xylenecyanol/0.05% bromophenol blue was added. The mixture was heated at 90°C for 1 min and layered on 12.5% (wt/vol) polyacrylamide/7 M urea gel (40 × 20 cm). The electrophoresis was performed in 50 mM Tris·borate, pH 8.3/1 mM EDTA. The individual cDNA product was characterized by two-dimensional electrophoresis/

Abbreviations: ddTTP, dideoxythymidine triphosphate; ddGTP, dideoxyguanosine triphosphate; bp, base pair(s).

homochromatography of its partial snake venon phosphodiesterase digest (16).

**Screening of cDNA Library.** The cDNA library was constructed from the cell line RPMI 4265 (the details to be published elsewhere). The clones were pooled into groups of 100–150 (total: 25 groups) and DNA from each group was prepared by CsCl/ethidium bromide equilibrium density centrifugation. DNA from each pool (2 μg) was digested with endonuclease *Hha* I. The digests were electrophoresed on 1.5% (wt/vol) agarose gel, followed by blotting to nitrocellulose filter paper as described by Southern (17). The blot was incubated with the $^{32}$P-labeled 30-nucleotide probe (20,000 cpm) in 25 ml of 0.45 M sodium chloride/0.045 M sodium citrate/0.02% polyvinylpyrolidone/0.02% Ficoll/0.02% bovine serum albumin/0.1% $NaDodSO_4$ at 50°C for 36 hr, followed by washing once with the mixture without $NaDodSO_4$ and twice with 0.030 M sodium chloride/0.030 sodium citrate, pH 7, at 50°C. The blot was subjected to autoradiography at −70°C. The colonies from the desired pool were streaked and hybridized with the same 30-nucleotide probe (18).

**Nucleotide Sequence of the cDNA Clone.** The plasmid DNA from the recombinant clone was digested with restriction endonuclease *Pst* I. The cDNA insert [approximately 1400-base-pairs (bp)-long, data not shown] was isolated by electrophoresis on 4% (wt/vol) polyacrylamide gel. The isolated DNA was digested with endonuclease *Sau* 96I followed by electrophoresis on 4% polyacrylamide gel. The largest fragment, about 700 bp long, was isolated, was labeled at its 5′ end with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP (19) and redigested with *Hin*fI. Two fragments ≈500 bp long and 200 bp long, labeled at one end, were isolated. The partial nucleotide sequence of the 500-bp fragment, starting from its 5′ end, was determined by the Maat–Smith procedure (20) and by the chemical degradation procedure (19).

## RESULTS AND DISCUSSION

Short, synthetic oligonucleotides can be synthesized relatively easily by either chemical (11, 14, 15, 21–23) or enzymatic methods (24). Such oligonucleotides have been used as primers for DNA (25) and RNA (26) sequence determinations, as hybridization probes to isolate cloned genes (27) and as detectors of specific mRNAs (28). We attempted to extend this approach to obtain cloned genes for the histocompatibility antigens, for which limited amino acid sequence information was available. While this work was in progress, Noyes *et al.* reported the use of a synthetic oligonucleotide derived from the amino acid sequence of gastrin to detect gastrin mRNA (29).

A portion of the amino acid sequence for the carboxyl end of the HLA-A2 and HLA-B7 antigens (30) and the deduced nucleotide sequence of the corresponding mRNA and oligodeoxynucleotide primer are shown in Fig. 1. The nucleotide sequence

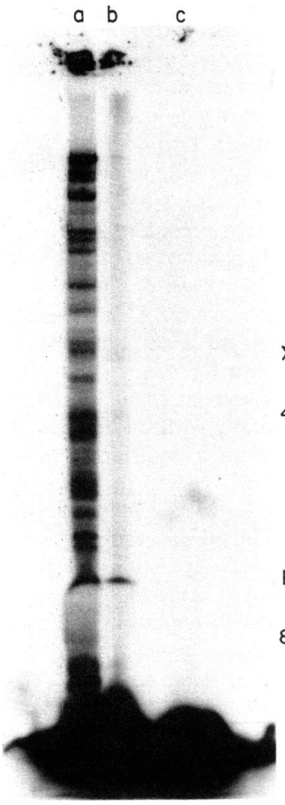


FIG. 2. Autoradiogram of the full-length cDNA synthesized by using the oligonucleotide primer and poly(A)-RNA. Channels: a, full-length synthesis with an oligonucleotide primer labeled at the 5′ end with $^{32}$P (specific activity, 1000–1500 Ci/mmol); b, full-length synthesis with a nonradioactive primer and [$\alpha$-$^{32}$P]dCTP (specific activity, 1 Ci/mmol); c, $^{32}$P-labeled primer only. The cDNA products were electrophoresed on a composite polyacrylamide/7 M urea gel, with the upper 2/3 of the gel containing 4% polyacrylamide and the lower 1/3 containing 8% polyacrylamide. XC and BPB represent the positions of xylene cyanol FF and bromophenol blue, respectively.

of the primer was derived from the tetrapeptide Met-Trp-Arg-Arg. The choice of the AGPU codon over the CGN codon for arginine was based on the known bias against the occurrence of the dinucleotide C-G in most, although not all, animal cell mRNA (32). Having made the choice of AGPU codon for arginine, we substituted T in the degenerate position in the complementary oligonucleotide primer because T can form a wobble base pair with G. The oligonucleotide primer was chemically synthesized by the triester method (14, 15) and was characterized by the two-dimensional mobility shift method (16) (data not shown). Fig. 2 shows the reverse transcriptase reaction performed with the primer on total poly(A)-mRNA with slight modification of the published procedure (33).

Because the oligonucleotide primer was complementary to the coding region and was situated approximately 90 nucleotides from the 3′ end of the coding region of the histocompatibility mRNA, the desired cDNA extension product was expected to be at least 1000 nucleotides long. Fig. 2 shows that there are several types of molecules in the size range anticipated for the histocompatibility cDNA. We believe that the large number of cDNA products are not the result of nonspecific degradation

HLA-B7 ($NH_2$)..... ( gly-ala-val-val-ala-ala-val)- met-cys-arg-arg-lys-ser-ser-gly-gly-lys-gly-gly-ser-tyr-ser-glu-ala-ala-

HLA-A2 ( $NH_2$)..... met-trp-arg-arg-lys-ser-ser-asp-arg-lys-gly-gly-ser-tyr-ser-glu-ala-ala

mRNA (5')..... GGN GCN GUN GUN GCN GCN GUN AUG UGG A C/G AC/A G−

OLIGONUCLEOTIDE

PRIMER (3')..... TAC ACC TCT TC

FIG. 1. Amino acid sequences of the two histocompatibility antigens HLA-B7 and HLA-A2 (30), the deduced sequence in the HLA-A2 or HLA-B7 mRNAs, and the nucleotide sequence of the oligonucleotide primer. The amino acid sequence in parentheses in HLA-B7 was available from R. Robb (31).

of mRNA, because the same pattern of bands was reproducibly obtained in several independent extensions by using different batches of poly(A)-mRNA. Our attempts to sequence the long cDNA products by the chemical degradation procedure (19) were only partially successful (data not shown) because of impurity and low radioactivity of the separated products.

There were at least 30 different cDNA products (Fig. 2). It is possible that the 11-nucleotide-long primer, which on statistical grounds is expected to find its complementary sequence approximately four times in the total sequence complexity of poly(A)-mRNA,* might have contained at least one mismatch within the arginine codons. This could make the 11-nucleotide primer equivalent to a decamer as regards the stability of mRNA·primer hybrid formation. Within the sequence of the primer, there are several possible decamer sequences that could form hybrids of comparable stability with the regions of mRNA coding for the tetrapeptide sequences different from Met-Trp-Arg-Arg.

To overcome this difficulty, a procedure was developed that reduced the heterogeneity and size of the cDNA products. This involved four separate reverse transcriptase reactions, each of which contained one of the four dideoxynucleoside triphosphates and the other three deoxynucleoside triphosphates (26). The products of the reactions were separated by electrophoresis in polyacrylamide/urea gels.

The principle of the method was as follows. The primer extension arising from the site of primer hybridization to a unique mRNA gave rise to four cDNA products of different chain lengths, each belonging to a particular dideoxynucleoside triphosphate-terminated reaction. The length of the product in each case was determined by the first occurrence of a nucleotide (in the mRNA) that was complementary to the dideoxynucleotide used. It then followed that one or more of the four cDNA products arising from primer hybridization to any given mRNA were represented among the cDNA products, which were four or more nucleotides longer than the primer. This was important, because the bands containing shorter-length cDNA products were expected to be less homogeneous. For this reason we did not need to analyze bands containing cDNA products that were respectively one, two, and three nucleotides longer than the primer.

For simplicity of further argument, we refer to the cDNA products that are four or more nucleotides larger than the primer as belonging to the "less heterogeneous fraction." The number of cDNA products resulting from primer extension from a unique mRNA, which would be represented in the less heterogeneous fraction, depended on the nature of the first three nucleotides (in the mRNA) proximal to the 3′ end of the primer at the site of primer hybridization. In a heterogeneous population of mRNAs such as poly(A)-mRNA, in which the primer hybridized to several mRNAs, the nucleotide sequence of the first three nucleotides proximal to the 3′ end of the primer could show the same relative representation of nucleotides as seen in the known genes. Assuming this randomness, we estimated that the less heterogeneous fraction of the cDNA products contained approximately 70% more cDNA products than would be obtained in the full-length cDNA synthesis reaction, in which primer extension from one mRNA gave rise to one cDNA product. However, because these products were divided into four dideoxy reactions, each dideoxy reaction would have 40% of the full-length cDNA synthesis products represented in its less heterogeneous fraction. Furthermore, because the inclusion of the dideoxynucleoside triphosphates in the reaction mixture greatly reduced the size of the cDNA extension products, the relative amounts of various cDNA products probably reflected more accurately the relative amounts of corresponding mRNAs. This was in contrast to the full-length cDNA synthesis reaction in which there could be a bias against the longer cDNA products either because of partial degradation of mRNA during isolation or premature termination of reverse transcriptase. The considerably reduced size of the cDNA products also increased the sensitivity of their characterization many fold.

Finally, from the knowledge of the nature of the amino acid preceding those that were used to deduce the primer sequence, one could limit the analysis of the cDNA products from one, two, or three of the four dideoxynucleoside triphosphate-terminated reactions.

In the case of histocompatibility antigens (Fig. 1), the amino acid preceding those used for deducing the primer sequence was valine with GUN codon in the mRNA. The cDNA products arising from the primer hybridization to the histocompatibility mRNA would be respectively 13 and 14 nucleotides long in the dideoxyadenosine and dideoxycytidine triphosphosphate-terminated reactions and, hence, would not be represented in the less heterogeneous fraction. Therefore, we analyzed the cDNA products from the dideoxyguanosine triphosphate (ddGTP)- and dideoxythymidine triphosphate (ddTTP)-terminated reactions. Furthermore, from the deduced mRNA sequence (Fig. 1), one could infer that the desired cDNA product might have been in the degenerate 15th position or in the 16th position in the ddGTP-terminated reaction (Fig. 3). Similarly, in the ddTTP-terminated reaction (Fig. 3), the desired cDNA product might have been in one of the degenerate positions (15, 18, 21, 24, 27, 30). Upon sequence determinations of these bands, we

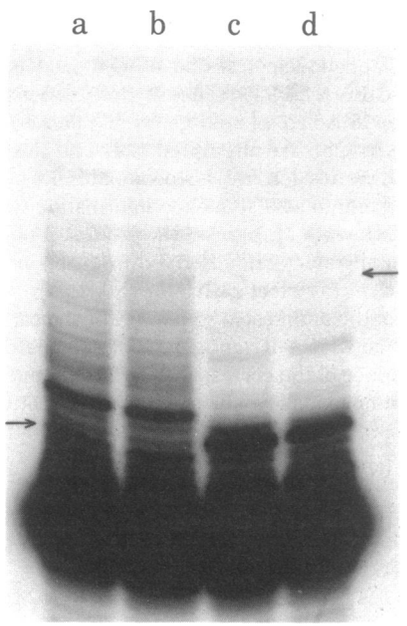


FIG. 3. Autoradiogram of the dideoxynucleoside triphosphate-terminated cDNA synthesis reactions. Channels: a and b, the reverse transcriptase reactions terminated by the ddGTP; c and d, the reverse transcriptase reactions terminated by the ddTTP. Arrows represent the positions of band 16 in the ddGTP channels and band 30 in the ddTTP channels. The reaction products were electrophoresed on 12.5% polyacrylamide/7 M urea gel.

* The size of the human genome is taken to be $2.6 \times 10^9$ bp, and 70% of it represents unique sequence DNA (34). It was assumed that nucleotide sequence complexity of mRNA from an adult-cell-type represents about 1% of the sequence complexity of single copy DNA, as was observed in the case of sea urchin (35). This figure comes to about $1.8 \times 10^7$ bp. The frequency of occurrence of a unique 11-nucleotide-long sequence is $1/4 \times 10^6$.

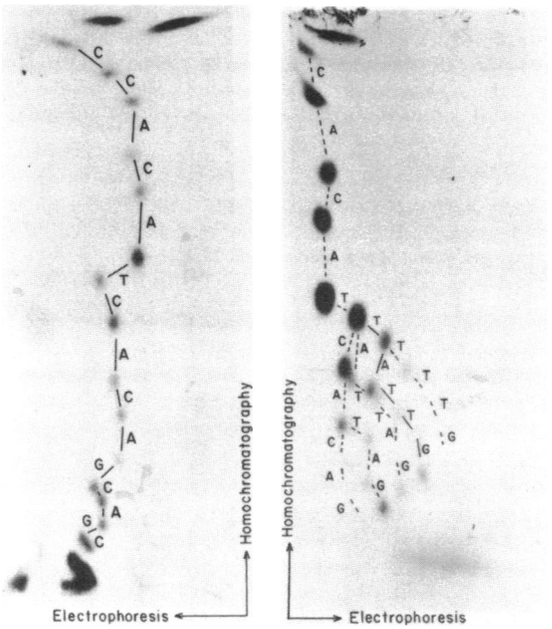


FIG. 4. Autoradiogram of the two-dimensional electrophoresis/homochromatographic analysis of the partial snake venom phosphodiesterase digest of the 30th position band from the ddTTP-terminated reaction (*Left*) and the 16th position band from the ddGTP-terminated reaction (*Right*). Homochromatography was performed with 75% Homo-B for 16 hr at 63°C in order to achieve better resolution of the longer-sized oligonucleotides of the partial digest. From the nature of the ddGTP-terminated reaction, a G residue was not expected between the 3′ end of the primer sequence and the 16th residue of the cDNA product. This was useful in drawing "branches," each representing a two-dimensional map of the individual cDNA product within the 16th position band. The left-end branch represents the sequence complementary to the histocompatibility mRNA.

found that the cDNA product at the 30th position in the ddTTP-terminated reaction and one of the cDNA products in the band at the 16th position in the ddGTP-terminated reaction did contain sequences potentially complementary to the histocompatibility mRNA (Fig. 4). The nucleotide sequence of the 30-nucleotide-long cDNA product was also determined by the chemical degradation procedure (19); it corresponded with the preliminary amino acid sequence of the HLA-B7 (data not shown). Although the nucleotide sequence of the primer was predicted to be complementary to the nucleotide sequence in HLA-A2 mRNA, the nucleotide sequence of the 30-nucleotide-long cDNA product turned out to be complementary to the HLA-B7 mRNA. Because the amino acid sequence of HLA-A2 antigen in this region is unknown, it is possible that the amino acid sequence of HLA-A2 antigen is identical to that of HLA-B7 antigen in this region and that the primer did extend, in fact, from hybridization to HLA-A2 mRNA. This argument is based on the evidence that HLA-A2 and HLA-B7 are highly homologous in their amino acid sequence (36). The alternative possibility that the 30-nucleotide-long cDNA product might have been the result of primer hybridization to HLA B7 mRNA, although less likely (see Fig. 1), cannot be ruled out.

We have also performed cDNA synthesis in the presence of three nucleoside triphosphates (by omitting the dideoxynucleoside triphosphate). Preliminary results suggest that the resulting cDNA products can be utilized either to directly sequence specific mRNAs from total poly(A)-RNA or to synthesize longer cDNA probes.

**Construction and Screening of cDNA Library.** The double-stranded cDNA was synthesized from poly(A)-mRNA as described (33). The plasmid pBR322 was digested with *Pst* I endonuclease, and homopoly(dG) tails were introduced at its 3′ ends by terminal transferase. The double-stranded cDNA derived from poly(A)-mRNA was likewise tailed with poly(dC). These two DNAs were hybridized, and the mixture was used to transform HB101 by the calcium chloride shock procedure. The resulting transformants were pooled into groups of 100–150 (total: 25 groups) and DNA from each group was prepared by CsCl/ethidium bromide equilibrium density-gradient centrifugation. The DNA from each pool was digested with the restriction endonuclease *Hha* I, followed by electrophoresis on 1.5% agarose gel. The Southern blots of these gels were hybridized to the 5′ end-labeled 30-nucleotide probe. Fig. 5 shows the result of hybridization to a Southern blot of DNAs from pools E to O. Only one DNA band from pool E revealed hybridization with the 30-nucleotide probe. The colonies from which pool E DNA was made were then screened with the same probe. Only one colony showed hybridization (data not shown). The plasmid DNA from this colony was obtained by equilibrium density centrifugation.

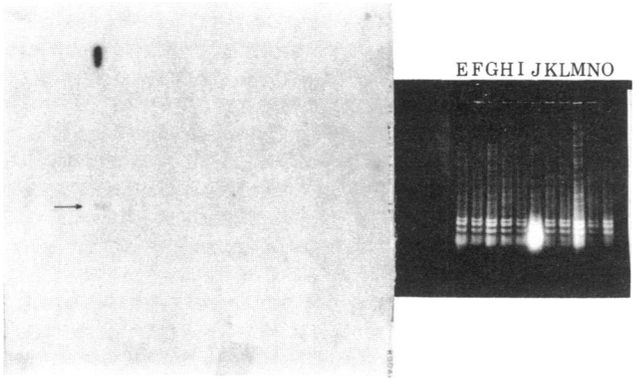


FIG. 5. Autoradiogram of the hybridization of the Southern blot with the $^{32}$P-labeled 30-nucleotide probe. DNAs from pools E to O were digested with the restriction endonuclease *Hha* I and were electrophoresed on 1.5% agarose gel. The Southern blot of this gel was hybridized to the 30-nucleotide probe. (*Left*) Only one DNA band (arrow) from pool E hybridized to the 30-nucleotide probe. (*Right*) The complexity of each pool of DNA before Southern blotting.

**Characterization of the cDNA Clone.** The recombinant plasmid was digested with *Pst* I endonuclease, and the cDNA insert was purified on 4% polyacrylamide gels. The size of the cDNA insert was estimated by electrophoreses alongside known size makers to be about 1400 bp (data not shown). The cDNA insert was digested with endonuclease *Sau* 96I, which gave a largest fragment ≈700 bp long and several small fragments ranging in length from <100 bp to ≈175 bp (data not shown). The 700-nucleotide-long fragment constituted the 3′ end of the cDNA clone (based on mapping data not shown). This fragment was isolated, labeled at its 5′ end with polynucleotide kinase and [$\gamma$-$^{32}$P]ATP, and redigested with *Hinf*I to give two fragments ≈500 and 200 bp long. The partial nucleotide sequence of the 500-bp-long fragment was determined by the Maat–Smith procedure (20) and by the chemical degradation procedure (19).

Fig. 6 shows one of the sequence-determination gels and the correspondence between the nucleotide sequence from this region of the cDNA clone and the amino acid sequence from position 223 to position 269 of the HLA-B7 (36). Except for the amino acid at position 242 (Glu), the nucleotide sequence corresponds to the amino acid sequence.

HLA-A and -B antigens comprise about 0.05–0.1% of total cellular protein of lymphoblastoid cell lines (37). Out of a library of 2500–3000 recombinant cDNA clones, we observed that the

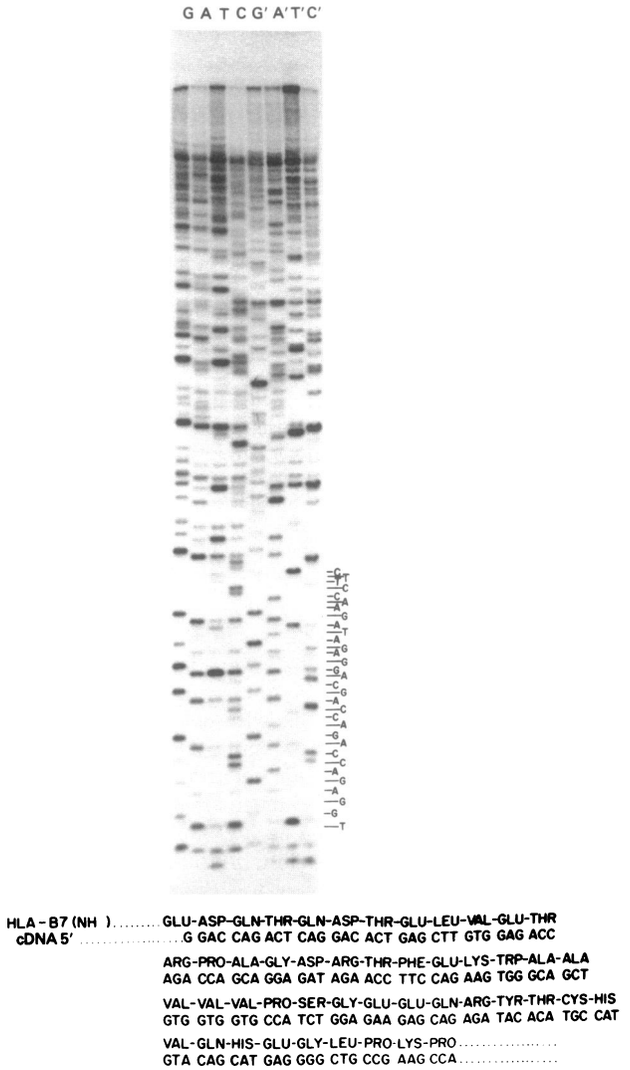


FIG. 6. Comparison of the amino acid sequence of HLA-B7 with the nucleotide sequence of the cDNA clone. The amino acid sequence starts from position 222 from the amino-terminal end of HLA-B7. The nucleotide sequence starts from the 5′ end of the *Sau* 96IA fragment. The autoradiogram shows one of the sequence-determination gels obtained by the Maat–Smith procedure (20). Lanes: G, A, T, C, reactions with standard procedure (19); G′, A′, T′, C′, reactions with only dideoxynucleoside triphosphates (modified method developed by P. Jagadeeswaran, personal communication). The right four lanes are useful in eliminating ambiguities at certain places.

30-nucleotide probe hybridized to only one cDNA clone, which we have characterized as HLA-B. From the HLA type of RPMI 4265 cell line (HLA-A2, A2, B7–B12), HLA-A2 cDNA clone was expected to be present twice more frequently than one of the HLA-B cDNA clones. These results suggest that an individual HLA mRNA constitutes about 0.01–0.05% of poly(A)-mRNA in rough agreement with protein data (37).

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