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THE HLA-E GENE ENCODES TWO DIFFERENTIALLY REGULATED TRANSCRIPTS AND A CELL SURFACE PROTEIN[1]

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An HLA-E-specific oligonucleotide probe was used to study the expression of HLA-E. This probe detects two HLA-E transcripts, 1.8 and 2.7 kb in size, which are present in varying ratios in all tissues and cell lines investigated. We demonstrate that alternative poly(A) site usage accounts for the differential regulation of the two HLA-E mRNA species. Sequence analysis of three cDNA clones, representing the two transcripts of HLA-E, and of an HLA-E gene encoded by cosmid cd3.14, revealed identity of gene and cDNA in the 3′ untranslated region. S1 nuclease protection assays confirmed that the two HLA-E transcripts are not alternative splicing products. Introduction of cd3.14, together with human β_2m into the murine myeloma cell line P3X63-Ag8.653, resulted in a cell surface expression of an HLA-class I heavy chain detectable by indirect immunofluorescence whereas transfection into the human β_2m expressing mouse L cell line, J27 was negative with regard to cell surface expression. Cell surface labeling of transfectants and immunoprecipitation with a monomorphic HLA class I-specific antibody or an antibody against human β_2m confirmed the presence of an HLA-E H chain on the cell surface. These results indicate that the HLA-E gene codes for a class I H chain that can be expressed on the cell surface.

The human MHC class I gene cluster located on the short arm of chromosome 6 comprises at least 17 genes, pseudogenes, and gene fragments (1). Among these, only the highly polymorphic, ubiquitously expressed classical class I loci HLA-A, -B, and -C encode serologically defined cell surface glycoproteins of approximately 44 kDa that are noncovalently associated with invariant β_2m[3] and that constitute the restriction elements for virus-specific and allospecific CTL. The function of nonclassical class I genes is not clear so far. However, it has been proposed that in the mouse some non-H-2 class I Ag may have a function in self-tolerance and T cell restriction (2). Studying the contribution of the human non-HLA-A, -B, and -C class I gene products (HLA-E and HLA-F) to cellular immunity has been hampered by the lack of efficient cell surface expression upon transfection (3, 4). Therefore, a knowledge of the tissue specificity of transcription and of the regulation of cell surface expression may be helpful in elucidating their possible physiologic functions.

Like the classical MHC class I loci, the HLA-E gene is highly transcribed in many tissues (4–6), but displays only little genetic variation. Several groups have isolated HLA-E-derived sequences that differ by few amino acids (4, 5, 7–12). Conflicting results have been obtained concerning the cell surface expression of the HLA-E-encoded class I heavy chain. Upon transfection of a 6.2-kb *Hin*dIII HLA-E subclone into an HLA class I negative mutant B-LCL, only an intracellular 41-kDa H chain could be coprecipitated with β_2m (3, 4). In contrast, the transfer of a 16-kb *Eco*R I subclone containing another HLA-E gene, was reported to give rise to detectable surface expression when transfected into mouse L cells that also express human β_2m (8). The controversial results concerning cell surface expression of HLA-E led us to introduce a genomic cosmid as well as a cDNA clone of HLA-E into mouse cell lines and to test stable transformants for the presence of HLA-E mRNA and cell surface expression.

Inasmuch as Northern blot analysis revealed two transcripts of HLA-E in all cell lines and tissues analyzed, we wondered whether they might encode different translation products. Therefore, we isolated cDNA clones and performed S1 nuclease protection experiments to determine the nature of the HLA-E transcripts.

MATERIALS AND METHODS

Tissue culture, human cell lines, and stimulation. All cells were cultured in RPMI 1640 supplemented with 10% FCS, 2% L-glutamine, and 1% penicillin/ streptomycin (all GIBCO, Grand Island, NY), with the exception of the L cell line, J27 which was maintained in DMEM HAT medium supplemented with 10% FCS, 2% L-glutamine, and 1% penicillin/streptomycin. The following human cell lines were used in this study: the B-LCL, LG2 (HLA-type: -A2, -B27, -Cw1) (13), TY (A11, -B35, -Cw4), and KR (-A2, -B44, -Cw5), RaDa and KaFraDa (both HLA-B27$^+$), the melanoma cell line Mel Juso, the chronic myelogenous leukemia cell line K562, the cervix epitheloid carcinoma cell line HeLa and the colon carcinoma cell line SW480. T-All are PBL of a patient suffering from acute T lymphatic leukemia. The cell lines m.m. and m.c. have been established by Dr. I. Funke, Munich, F.R.G. from bone marrow micrometastases of a patient with breast carcinoma (m.m.) and colon carcinoma (m.c.), respectively.

The 4 to 5 × 10^6/ml K562 and SW480 cells were stimulated with 1000 U/ml of human rIFN-γ (Boehringer Mannheim, Indianapolis, IN) for 48 and 24 h, respectively.

PBL were isolated and cultivated according to standard protocol.

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[3] Abbreviations used in this paper: β_2m, β_2-microglobulin; B-LCL, B lymphoblastoid cell line; aa, amino acid; 3′ UT, 3′untranslated region; AGPT, aminoglycoside phosphotransferase; PCR, polymerase chain reaction; CHX, cycloheximide.

PHA (Sigma) was added at a concentration of 2 μg/ml. After incubation for 2 days, the PBL culture was split into two fractions and CHX (Sigma) was added at a concentration of 10 μg/ml (2 h) to one fraction.

Murine cell lines and transfection. The L cell line, J27 (14), which expresses human β_2m was transformed by the calcium phosphate-mediated DNA transfer technique with either cosmid cd3.14 or the expression cDNA construct, pLG2-C1/KSVneo, both clones carrying the AGPT gene. Transformants were selected and grown in DMEM HAT with 1 mg/ml G418 (GIBCO). The mouse myeloma cell line P3X63-Ag8.653 (X63) was cotransfected by electroporation (15) with a *Bam*H I linearized 15-kb *Bam*H I/*Sal*I subclone of the human β_2m-gene in pUC18 (16) together with either a *Sal*I linearized 6.3-kb *Eco*R I/*Bam*H I subclone of the cosmid cd2.6 (17) in pTM or with the *Cla*I linearized cosmid cd3.14, the latter both carrying the AGPT gene. Transfected cells were selected and grown in RPMI 1640 containing 1 mg/ml G418 (GIBCO).

Monoclonals. Hybridomas producing mAb specific for human β_2m class I heterodimers, B9.12.1(16), W6/32, and TM3 (18); human β_2m, BBM.1; H-2K^k, 141-11, and H-2D^d, 34-4-21S (19) were kindly made available by the respective laboratories. The mAb specific for human class I H chain, A1.4 ((20); United Biomedical Inc., Hauppauge, NY) and human CD4, M-T242 were used as purified antibody.

Indirect immunofluorescence. The 4×10^5 cells were incubated with 40 μl of hybridoma supernatant or A1.4 at a concentration of 20 μg/ml for 30 min on ice. Cells were washed twice with PBS and then incubated with 40 μl dichlorotriazinyl aminofluorescein goat anti-mouse Ig (Dianova, Hamburg, F.R.G.) at a concentration of 25 μg/ml for 30 min on ice. After four washes with PBS, samples were fixed with 1% paraformaldehyde in PBS and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Isolation of cd3.14, LG2-C1,-E1, and -E2. A genomic cosmid library (cd) established from PBL of a healthy donor (HLA-type: -A2, -B27/-B51, -Cw2/-Cw3) (17) was screened for class I$^+$ clones with the HLA-B cDNA probe pB1 (13). Eleven distinct class I genes were rescreened using the oligomer oLG2-C1 (aa 143 to 153, 33 bp): 5′-CGC CTC AGA GGC ATC ATT TGA CTT TTG CTC GGA-3′, unique for the HLA-E sequence, which hybridized only to cosmid cd3.14. Although the HLA-E allele encoded by cosmid cd3.14 carries a silent substitution in the triplet aa 150, GCT instead of GCC, the length of 33 bp of the oLG2-C1 oligonucleotide explains why this difference was not discriminated at the hybridization and washing conditions applied. The isolation of LG2-C1 from a cDNA library constructed from the homozygous B-LCL LG2 (13) has been described elsewhere (7). The clones LG2-E1 and LG2-E2 were obtained from the same cDNA library using a 3′ 2.8-kb *Bam*HI fragment of cd3.14 as the probe (Fig. 2). By in vitro mutagenesis and several subcloning steps, the incomplete 5′ end of the LG2-C1 cDNA was replaced by the 5′ flanking and exon 1 sequence of an HLA-B8 cDNA (7) and subcloned as *Bam*H I fragment into the *Bgl*II site of the eucaryotic expression vector pKSV10 (Pharmacia Fine Chemicals, Piscataway, NJ). The nucleotide sequence of the generated 5′ end, including the codon for the first amino acid residue of the mature protein, is: GGATCCACCCTTAAGATA ATG CTT GTG GGG GCG CGC CGA ACC GTC CTC CTG CTG CTC TCG GGA GCC CTG ACC GAG ACC TGG GCC TGG GCG *GGC*. As a selection marker, a *Bam*HI/*Sal*I fragment containing the AGPT gene, was inserted into the *Bam*HI/*Sal*I sites of this subclone, generating pLG2-C1/KSVneo.

DNA sequence analysis. DNA sequence analysis of M13 subclones or the 2.8-kb *Bam*HI fragment subcloned into Bluescript (Stratagene, La Jolla, CA) was performed according to the sequencing strategy depicted in Figure 2. The region carrying exon 2 and 3 of cosmid cd3.14 was directly sequenced using the primers described by (10) and oLG2-C1. Dideoxy chain termination method was applied using the T7 sequencing kit of Pharmacia.

Southern and Northern blot analysis. Southern blot analysis of genomic DNA isolated from various HLA haplotypes was carried out according to a standard protocol. Probes were labeled by the random primer method (21) with [α-^{32}P]dATP (Amersham Corp., Arlington Heights, IL) and hybridization was performed according to the method of Church and Gilbert (22). Purification of the 33 mer oLG2-C1 and hybridization at 65°C were performed as described (23). The probe was labeled with terminal desoxynucleotidyl transferase (Boehringer Mannheim, Indianapolis, IN) and [α-^{32}P]dATP (Amersham) (24). Washing was done at 65°C with 3x SSC, 10 mM NaH_2PO_4/Na_2HPO_4, 10x Denhardt's, 5% SDS for 2x 20 min and with 1x SSC, 1% SDS for 10 min.

RNA from human tissue, cell lines, and PBL was prepared by the procedure of Chirgwin et al. (25). A total of 10 to 20 μg of total RNA was separated on formaldehyde agarose gels and transferred to Hybond-N membrane (Amersham). The filters were hybridized with the 3′ 2.8-kb *Bam*HI fragment of cd3.14, a 3′ UT 450-bp HLA-B-specific *Pst*I/*Pvu*II probe (13) and a 2.8-kb human β_2m specific *Bgl*II fragment (16). Hybridization with oLG2-C1 and with the c-fos-specific oligomer c-fos/o1 (aa 4–24, 60 bp): 5′-C CCC GGC CGG GGA CGC GCT GCT GCA GCG GGA GGA GTA CGC CTC GTA GTC GTT GAA GC-3′ and subsequent washing were performed as described above at 65°C.

Generation of single stranded probes by PCR (Fig. 5B). Linear PCR amplification of 50 ng of a subclone of LG2-C1 in pUC18, digested with *Eco*R I, was performed in 10 cycles successive to an initial denaturation step at 94°C for 2 min with the following conditions: 94°C (2 min) and 72°C (2 min); the last extension reaction (72°C) was extended for 5 min. The reaction was primed with 30 ng of purified oligonucleotide oLG2-C1 generating a single stranded antisense probe, 534 nucleotides in length.

A total of 50 ng of the same subclone, digested with *Ava*II, was amplified in 10 cycles succeeding an initial denaturation step at 94°C for 3 min adopting the subsequent conditions: 94°C (1 min), 56°C (1 min), and 72°C (2.5 min); the last extension reaction (72°C) was 5 min. As primer 25 ng of a 21 mer located at the 3′ end of exon 4 (aa 262–269: 5′GCT CGG GTA GCC CCT CAT GCT 3′) was used. The size of the single stranded antisense probe was 404 nucleotides. PCR amplification (26) was carried out in 20 μl of a buffer containing 50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1 mM Mg_2Cl, 100 μM dGTP and dTTP, 90 μM dATP and dCTP, and 3 μl each of [α-^{32}P]dATP and [α-^{32}P]dCTP (both Amersham 370 MBq/ml, 3.33 μM) with 1.25 U Taq polymerase (Boehringer-Mannheim) in a PCR Thermal Reactor (Hybaid, Teddingbon, U.K.).

The probes were passed over a Sephadex G50 spin column and purified by electrophoresis through a 4% polyacrylamide/7 M urea gel. The bands of the correct size were excised and eluted at 37°C for 3 h in 500 μl 500 mM ammonium acetate, 0.5% SDS, and 1 mM EDTA. After a phenol/chloroform extraction step, three aliquots of each probe were precipitated with 30 μg of the RNA preparations as described below.

S1 nuclease protection assays. The conditions for hybridization and S1 nuclease digestion were as described (27) with the following modifications. Three 30-μg samples of each total RNA were hybridized overnight with equal aliquots of either of the continuously labeled probes (sp. act. ~10^7 cpm/μg) in a volume of 20 μl at 32°C. Digestion was performed at 32°C for 2 h with 600, 1200, or 2400 U of S1 nuclease for each assay. The protected DNA was precipitated and redissolved in 4 μl of 10 mM Tris-U, pH 8.0, 1 mM EDTA, pH 8.0, and analyzed by electrophoresis through a 4% polyacrylamide/7 M urea gel.

cDNA synthesis and PCR. For first strand cDNA synthesis 2 μg of total RNA were denaturated 3 min at 94°C in 5 μl of 5 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol plus 12 ng/ml oligo dT. After an annealing step for 10 min at 50°C the reaction volume was raised to 10 μl with the same buffer plus 5 μM dNTP, and 200 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL, Gaithersburg, MD), and cDNA synthesis carried out for 60 min at 37°C. Each cDNA reaction sample was diluted 1/3 and 1 μl was amplified in 20 μl PCR buffer (50 mM KCl, 10 mM Tris-Cl, pH 8.3, 1 mM $MgCl_2$), 100 μM dNTP, 1.25 U Taq polymerase (Boehringer-Mannheim) and 50 ng each of the amplification primers P1 and P2 (Fig. 6). P1 (5′-CAG CAT GAG GGG CTA CCC G-3′) is a sense oligo specific for the 3′end of the exon 4 of HLA-E. P2 (5′-GTG TGA GGA AGG GGG TCA TG-3′) is an antisense oligo derived from the 3′untranslated region of the HLA-E mRNA. The following amplification was adopted using a PCR Thermal Reactor (Hybaid): 5 min, 94°C; 10 cycles: 1 min 94°C, 1 min 50°C, 1 min 72°C; 40 cycles: 1 min 94°C, 1 min 55°C, 1 min 72°C; 10 min 72°C. The PCR products were analyzed on a 1.5% agarose gel containing ethidium bromide.

Preparation of radiolabeled proteins. The iodination of cell surfaces was carried out using the lactoperoxidase-glucose oxidase method (28). In brief, 3×10^7 cells cultured at 37°C were washed twice with PBS and labeled in 1 ml of PBS with 5 U lactoperoxidase (Sigma, L-0515), 6 U glucose oxidase type V (Sigma), and 1 mCi of carrier free Na ^{125}I (17.4 Ci/mg; Du Pont, Wilmington, DE) in the presence of 0.01 mM KI and 5 mM D-glucose for 20 min at room temperature. Cells were washed five times in PBS/10% FCS and lysed.

Immunoprecipitation and SDS-PAGE. Cells were lysed at 4°C for 30 min in 0.6 ml lysis buffer (1% (w/v) Nonidet-P40, 2 mM benzamidine HCl, 2 mM PMSF, 2% (v/v) aprotinin in PBS). After sedimentation of nuclei and debris at 15,000 × *g* for 15 min at 4°C, lysates were suppemented with 100 μl of FCS and precleared at 4°C 5 x 12 h with 100 μl horse serum saturated Sepharose 6B (Pharmacia) and 2 x 12 h with 50 μl of protein A-Sepharose CL-4B (Sigma).

^{125}I-labeled proteins were sequentially immunoprecipitated (1 h at 4°C) using the following reagents: 20 μl isotype control M-T242-protein A-Sepharose, 20 μl of A1.4-protein A-Sepharose and finally 20 μl of BBM.1-protein A-Sepharose. Between each round lysates were cleared with 20 μl of protein A-Sepharose for 2 h at 4°C.

Immunoadsorbents were washed six times in PBS containing 0.2% (w/v) Nonidet-P40, resuspended in 50 μl of SDS sample buffer (4.2% SDS, 0.1% 2-ME, 18% glycerol), boiled for 3 min, and analyzed by SDS-PAGE on 10% gels.

RESULTS

Invariant structure of HLA-E gene. Comparison of the sequence of the HLA-E cDNA clone LG2-C1 (7) to the classical HLA-A, -B, and -C genes reported to date, showed the greatest divergence to be clustered in a stretch of DNA encoding the amino acid residues 143 to 153. To study HLA-E gene expression, an oligonucleotide oLG2-C1 corresponding to this region was synthesized and shown to be strictly specific for the HLA-E gene in Southern blot analyses of genomic DNA restricted with *Bgl*II and *Pst*I, which give rise to fragments characteristic of HLA-E (Fig. 1). This oligomer also identified a cosmid cd3.14 isolated from the cd library (17), established from PBL of a healthy donor, to contain an HLA-E gene. This assignment was confirmed by Southern blot and sequence analysis. Cosmid cd3.14 encodes the HLA-E allele, E*0105 (EMBL: X64880) with Gly at aa positions 83 and 107 compared to the HLA-E allele E*0102, encoded by HLA-6.2 (4, 11) and a silent substitution in the triplet for aa 150, GCT instead of GCC. The restriction map of cd3.14 differs from that of the HLA-6.2 allele by a 3′ *Bam*HI restriction site and from the HLA-E gene encoded by cosmid RS5 (8, 9) by a *Pst*I (aa 95) and a *Bgl*II site (3′untranslated region) within the HLA-E gene, in addition to sites upstream and downstream of the coding region (Fig. 2). Southern blots of *Pst*I, *Bgl*II, and *Bam*H I digested genomic DNA from various donors with different haplotypes hybridized with either oLG2-C1 (Fig. 1), or with subfragments of the HLA-E cDNA clones (Fig. 2), a 3′ 950 bp *Pst*I fragment of LG2-C1 (data not shown) or with a 400-bp *Pst*I/*Pvu*II fragment of the LG2-E2 cDNA clone (data not shown) confirmed the restriction map of cd3.14 and did not show a restriction fragment length polymorphism.

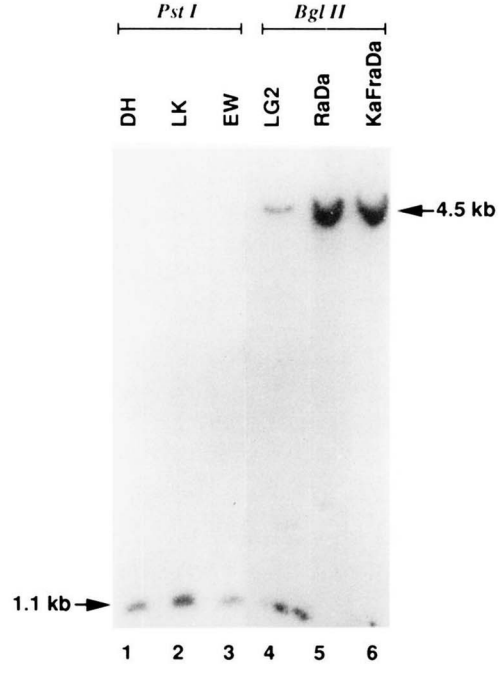

Figure 1. Southern blot analysis confirms the organization of the HLA-E gene. Genomic DNA isolated from PBL (*DH, LK, EW*) of donors with various HLA haplotypes and from B-LCL (*LG2, RaDa, KaFraDa*) sharing the B27 allele, was digested with *Pst*I and *Bgl*II, generating fragments unique to the HLA-E gene, and probed with oLG2-C1.

HLA-E gene transcription does not display tissue restriction. Northern blots hybridized with HLA-E subfragments revealed two transcripts (4, 5, 12). To investigate the tissue expression of HLA-E mRNA and the ratio of the two transcripts, an antisense oligomer, oLG2-C1, specific for both mRNA, was used. Considerable amounts of two transcripts of HLA-E of 1.8 and 2.7 kb, respectively, were detected in various human cell lines of different origins and human tissues (Figs. 3, 5*A* and 7). The tissue distribution of HLA-E mRNA is not restricted and thus, resembles the expression pattern of the classical HLA-A/-B/-C genes and also the amount of HLA-E transcripts is similar (Fig. 3). Even in unstimulated chronic myelogenous leukemia, K562 cells, known to be MHC class I$^-$, HLA-E mRNA can be detected after prolonged exposure of autoradiographs (Figs. 3, 5*A*, and 8*a*) (5). Interestingly, also in placenta more HLA-E mRNA was present than HLA-A or HLA-B. In all fresh tissues analyzed the larger transcript seemed to predominate with the exception of the lung, where the ratio of the two mRNA species was about one to one. In the cell lines examined to date, we found at least equivalent amounts of the two messages with a prevalence of the smaller transcript, except in T-ALL cells (PBL of a patient suffering from acute T lymphatic leukemia), in the cervix epitheloid carcinoma cell line, HeLa (Fig. 5*A*), and in the colon carcinoma cell line SW480 (Fig. 7*B*). Upon stimulation of PBL with PHA (Fig. 7*A*), an overall increase of HLA-E mRNA and a shift from the large transcript to the smaller mRNA was observed. This was also seen in the cell line SW480 treated with IFN-γ (Fig. 7*B*). This differential enhancement of the two HLA-E mRNA seems not to be a common feature of HLA-E induction, since K562 cells stimulated with IFN-γ (Fig. 5*A*) showed an equal increase of both transcripts.

Two HLA-E transcripts only differ in extent of 3′UT. It was of interest to determine whether the differential regulation of HLA-E transcripts might be important in the control of cell surface expression of HLA-E Ag, whether only the larger transcript is functional, and whether alternative splicing might produce a different translation product. A 3′ 2.8-kb *Bam*H I fragment from cd3.14 (Fig. 2) hybridized exclusively to the large transcript (data not shown) and was used to isolate the cDNA clones LG2-E1 (Figs. 2 and 4) and LG2-E2 (Fig. 2, codes for the sequence 848 to 1557 bp, Fig. 4) from the LG2 library, established from the B lymphoblastoid cell line LG2 (13). Sequence analysis of LG2-E1 (-E2) as well as of the 3′ region of cd3.14 revealed identity of the cDNA and the genomic sequences (Figs. 2 and 4). Two differences were found in LG2-E1 that might be explained by a cDNA cloning artefact (Fig. 4). No alternative splicing in the 3′ region was detected. A third polyadenylation signal is present 850 bp down-stream of two immediately adjacent sites and is used in the 2.7-kb transcript (Fig. 4). A partial HLA-E cDNA clone, similar to LG2-E1, E015, presumed to be derived from the 2.7-kb HLA-E mRNA, has recently been cloned from blood eosinophils (12). Several base substitutions are present when comparing our sequences to E015. The length of these cDNA confirm the size of 2.7 kb measured for the larger transcript.

Inasmuch as no full length cDNA clone coding for the

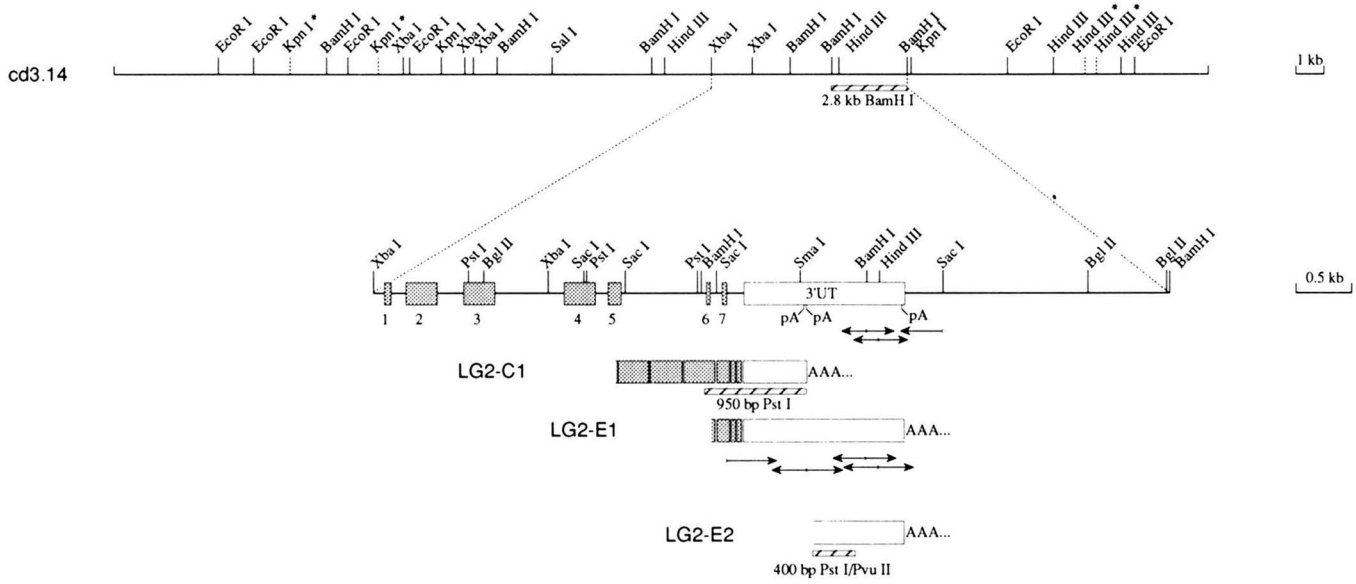

Figure 2. Restriction map of cosmid clone cd3.14 and cDNA clones LG2-C1, LG2-E1, and LG2-E2. The gene carrying region is shown as a blow-up. *Shaded boxes* numbered below indicate the coding exons 1–7. The 3′ UT is depicted as *open box*. *pA* designates the polyadenylation signals. The sequencing strategy is indicated by *arrows*. Restriction endonucleases sites with uncertain relative positions to each other are designated by *asterisk*. The origin and the location of the probes used in this study are indicated by *hatched boxes*.

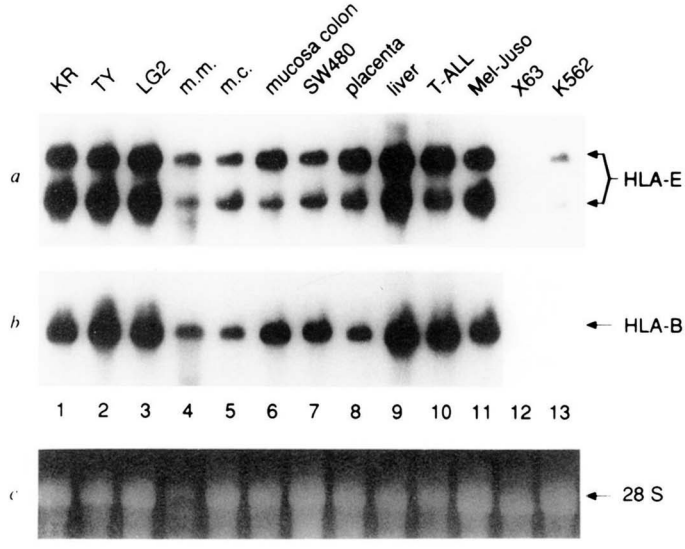

Figure 3. Detection of HLA-E mRNA by northern blot analysis. A total of 10 μg of total RNA per lane were separated on a formaldehyde agarose gel and probed with either oLG2-C1 (*a*) or with the 3′ UT 450-bp HLA-B-specific *Pvu*II/*Pst*I probe (*b*). The amount of RNA loaded is visualized by the ethidium bromide staining of the 28 S rRNA (*c*).

2.7-kb HLA-E transcript was obtained, RNA isolated from IFN-γ-induced K562 cells was used to investigate possible differences in the coding regions of the two HLA-E transcripts by S1 nuclease mapping. K562 cells stimulated for 48 h with 1000 U/ml IFN-γ showed high and equal amounts of both HLA-E messages, but only low level of HLA-B mRNA (Fig. 5*A*). Two overlapping probes derived from the cDNA clone LG2-C1, covering the sequence coding for the leader up to the end of the α3 domain, were used (Fig. 5*B*). Only a single band of the expected size was detected with either of the two probes in K562 RNA, demonstrating that both HLA-E transcripts are identical in the 5′ portion (signal sequence up to the 3′end of exon 4). The bands were shown to be specific for HLA-E, because they are not protected in HLA-B27$^+$X63 transfectants. Identity of the 3′ portion of the HLA-E transcripts was confirmed by PCR amplification, using primers located in exon 4 and the 3′ UT (Fig. 6). HLA-E mRNA isolated from the L cell and X63 transfectants, the human cell lines K562, Jurkat, LG2 and the human tissues, spleen, liver, thymus, and placenta gave rise to the same amplification product of the expected size of 447 bp (Fig. 6). These results demonstrate that the two HLA-E transcripts code for the same polypeptide and differ only in the extent of the 3′ untranslated region.

Differential regulation of two HLA-E mRNA species. Interestingly, two AUUUA motifs that confer instability to the mRNA of certain lymphokines and oncogenes, including c-fos (29, 30), are located downstream of the first two immediately adjacent polyadenylation signals in the HLA-E gene and are present only in the large transcript (Fig. 4). Thus, it was conceivable that a rapid degradation specifically of the 2.7-kb mRNA in activated cells, directed by this element, might explain the prevalence of the 1.8-kb HLA-E mRNA upon stimulation. To test this possibility, PBL were activated with PHA in the presence or absence of the inhibitor of protein synthesis, CHX, which leads to the stabilization of mRNA molecules sensitive to this specific degradation. Inasmuch as the two HLA-E transcripts share the same transcriptional start point, differential regulation at the level of transcription initiation that might equally be affected by CHX, can be ruled out. As shown in Figure 7*A*, CHX increases the steady-state levels of both HLA-E transcripts. Similarly, c-fos mRNA, investigated as positive control, accumulates drastically upon PHA/CHX treatment because CHX prevents the enhanced degradation of c-fos mRNA during prolonged stimulation (29). The same result was obtained with Jurkat cells. Increased levels of both HLA-E transcripts upon 12-*O*-tetradecanoylphorbol 13-acetate/PHA stimulation were further amplified by CHX treatment, without affecting the ratio of the two messages (data not shown). Therefore, we conclude that differences in stability of the two HLA-E transcripts do not account for the prevalence of the 1.8-kb mRNA upon stimulation.

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     EXON 4-Alpha 3                                                                                    EXON 5-Transmembrane
  1  A TAC ACG TGC CAT GTG CAG CAT GAG GGG CTA CCC GAG CCC GTC ACC CTG AGA TGG A  AG CCG GCT TCC CAG CCC
       Tyr Thr Cys His Val Gln His Glu Gly Leu Pro Glu Pro Val Thr Leu Arg Trp   Lys Pro Ala Ser Gln Pro
       257                                                                  274   275                 280
 74  ACC ATC CCC ATC GTG GGC ATC ATT GCT GGC CTG GTT CTC CTT GGA TCT GTG GTC TCT GGA GCT GTG GTT GCT GCT
     Thr Ile Pro Ile Val Gly Ile Ile Ala Gly Leu Val Leu Leu Gly Ser Val Val Ser Gly Ala Val Val Ala Ala
     281                                                        296                                 305
                                      EXON 6-Cyto 1                                      EXON 7-Cyto 2
149  GTG ATA TGG AGG AAG AAG AGC TCA G  GT GGA AAA GGA GGG AGC TAC TCT AAG GCT GAG T  GG AGC GAC AGT GCC
     Val Ile Trp Arg Lys Lys Ser Ser   Gly Gly Lys Gly Gly Ser Tyr Ser Lys Ala Glu   Trp Ser Asp Ser Ala
     306                          313   314                                    324   325             329
                                          EXON 8
221  CAG GGG TCT GAG TCT CAC AGC TTG TAA AG  CCTGAGACAGCTGCCTTGTGTGCGACTGAGATGCACAGCTGCCTTGTGTGCGACTGAGAT
     Gln Gly Ser Glu Ser His Ser Leu  *
     330                          337
311  GCAGGATTTCCTCACGCCTCCCCTATGTGTCTTAGGGGACTCTGGCTTCTCTTTTTGCAAGGGCCTCTGAATCTGTCTGTGTCCCTGTTAGCACAATGTG
411  AGGAGGTAGAGAAACAGTCCACCTCTGTGTCTACCATGACCCCCTTCCTCACACTGACCTGTGTTCCTTCCCTGTTCTCTTTTCTATTAAAAATAAGAAC
511  CTGGGCAGAGTGCGGCAGCTCATGCCTGTAATCCCAGCACTTAGGGAGGCCGAGGAGGGCAGATCACGAGGTCAGGAGATCGAAACCATCCTGGCTAACA
611  CGGTGAAACCCCGTCTCTACTAAAAAATACAAAAAATTAGCTGGGCGCAGAGGCACGGGCCTGTAGTCCCAGCTACTCAGGAGGCGGAGGCAGGAGAATG
711  GCGTCAACCCGGGAGGCGGAGGTTGCAGTGAGCCAGGATTGTGCGACTGCACTCCAGCCTGGGTGACAGGGTGAAACGCCATCTCAAAA AATAAA AAT
809  TAAAA AATAAA AAAAGAACCTGGATCTCAATTTAATTTTTCATATTCTTGCAATGAAATGGACTTGAGGAAGCTAAGATCATAGCTAGAAATACAGAT
907  AATTCCACAGCACATCTCTAGCAAATTTAGCCTATTCCTATTCTCTAGCCTATTCCTTACCACCTGTAATCTTGACCATATACCTTGGAGTTGAATATTG
1007 TTTTCATACTGCTGTGGTTTGAATGTTCCCTCCAACACTCATGTTGAGACTTAATCCCTAATGTGGCAATACTGAAAGGTGGGGCCTTTGAGATGTGATT
1107 GGATCGTAAGGCTGTGCCTTCATTCATGGGTTAATGGATTAATGGGTTATCACAGGAATGGGACTGGTGGCTTTATAAGAAGAGGAAAAGAGAACTGAGC
1207 TAGCATGCCCAGCCCACAGAGAGCCTCCACTAGAGTGATGCTAAGTGGAAATGTGAGGTGCAGCTGCCACAGAGGGCCCCCACCAGGGAAATGTCTAGTG
1307 TCTAGTGGATCCAGGCCACAGGAGAGAGTGCCTTGTGGAGCGCTGGGAGCAGGACCTGACCACCACCAGGACCCCAGAACTGTGGAGTCAGTGGCAGCAT
1407 GCAGCGCCCCCTTGGGAAAGCTTTAGGCACCAGCCTGCAACCCATTCGAGCAGCCACGTAGGCTCGACCCAGCAAAGCCACAGGCACGGGGCTACCTGAG
1507 GCCTTGGGGGCCAATCCCTGCTCCAGTGTGTCCGTGAGGCAGCACACGAAGTCAAAAGAGATTATTCTCTTCCCACAGATACCTTTTCTCTCCCATGACC
1607 CTTTAACAGCATCTGCTTCATTCCCCTCACCTTCCCAGGCTGATCTGAGGTAAACTTTGAAGTAA AATAAA AGCTGTGTTTGAGCATCATTTGTATTT
1705 CATTTGTGCGTTTTGTGCCTTGTTGTTTTAATTTTTTAACCACATTCAAGCTATCCTTTGGCTTCCAATGCCATGGTCCACCCAGAACTGCATTCACTGG
1805 CCCGTGTTCTAGTTCTGGTCATGCCGACTTTCCCGTTTTCCTGGTGAATCCCTGTAATCACCTGAGTCTCATTCTGTCAGGTGATATCCAGTAAGAAGGC
1905 AACATGTGCGGTGAGAAAGCCCAGGGAGTCCTGGGTGTGAATTTTTACTTTGCCATTTCTTCCTGTGTGTACACGCGGTGGGGCTTCACCTGTCTGAGCTC
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Figure 4. Nucleotide and deduced amino acid sequence of the large HLA-E transcript and the 3′ portion of the HLA-E gene. The nucleotide sequence of the protein coding region is given in triplets with the amino acid sequence below in the three letter code. The exons and corresponding protein domains are indicated above the sequence with spacing between individual exons. The numbers of the amino acids are shown for the first and the last residue of each exon and start with Tyr, the first amino acid encoded by LG-E1. The nucleotide numbering indicated on the left corresponds to the cDNA sequence contained in LG2-E1 up to the **C** at position 1695 and continues up to the *Sac*I site with the genomic sequence derived from the cd3.14 HLA-E gene. The sequences of the 3′ portion of cd3.14 and of LG2-E1 are colinear and identical, except for a substitution (A for G) at position 1218 and a deletion at position 1580 in LG2-E1 (indicated by bold type). Polyadenylation signals are underlined. AUUUA motifs reported to confer mRNA instability to oncogene and cytokine trancripts are shaded. The beginning of each exon is underlined. cDNA clone LG2-E2 codes for the sequences 848 to 1557 bp. (These sequence data are available from EMBL under accession number X64879.)

Expression of HLA-E Ag upon DNA transfer into mouse cell lines. Inasmuch as no HLA-E specific mAb is available, we used mouse cell lines to address the question whether HLA-E encodes a cell surface protein. Initially, mouse L cells expressing human β_2m (J27, (14)) were transfected either with the cosmid cd3.14 or with the cDNA construct pLG2-C1/KSVneo, encoding the 1.8-kb HLA-E cDNA, LG2-C1, with a HLA-B8-derived leader sequence. Unrearranged integration of the input DNA was confirmed by Southern blot analysis. All transfectants tested transcribe the HLA-E gene predominantly in a 1.8-kb mRNA (Fig. 8*B*), but no cell surface expression of an HLA class I H chain could be detected (Fig. 9). The same negative results were obtained on cotransfection of either HLA-E construct, together with the human β_2m gene, into mouse P815 cells.

In contrast, cotransfection of the cd3.14 cosmid and the human β_2m gene into the mouse myeloma cell line P3X63Ag8.653 (X63) resulted in a reproducible low level of cell surface expression of an HLA class I H chain in six independently isolated X63 transfectant clones, which transcribed both the human β_2m and the HLA-E genes (Figs. 8*a* and 9). Moreover, a transfectant clone receiving only HLA-E DNA also showed cell surface expression. Surface staining could be detected with all HLA-class I specific antibodies used: A1.4, B9.12.1 (Fig. 9), and TM3. W6/32 reacted already strongly with the single X63/human β_2m transfectants and was not used in further analyses. The cell surface expression was poor in all transfectants irrespective of the amount of HLA-E mRNA present and was much lower than the HLA-B27 staining when transfectant clones expressing similar levels of HLA-class I mRNA were compared (Figs. 5*A*, 8*a*, and 9). The cell surface expression of an HLA-E α-chain complexed with β_2m was confirmed by labeling membrane proteins with ^{125}I in lactoperoxidase-catalyzed reactions and subsequent immunoprecipitation with either BBM.1 or A1.4 (Fig. 10), detecting a 42-kDa H chain in the HLA-E transfectants. In agreement with the staining of the transfectants by indirect immunofluorescence, the amount of labeled HLA-E encoded H chain was much lower than that of the HLA-B27 α-chain in the corresponding transfectant. Immunoprecipitation of metabolically labeled X63-transfectants also showed that the amount of labeled HLA-E encoded H chain was much lower than that of the HLA-B27 α-chain (which contains the same number of methionine residues as the HLA-E polypeptide) in the corresponding transfectant, indicating that the low HLA-E cell surface expression is due to reduced levels of HLA-E polypeptide within the cell (30a).

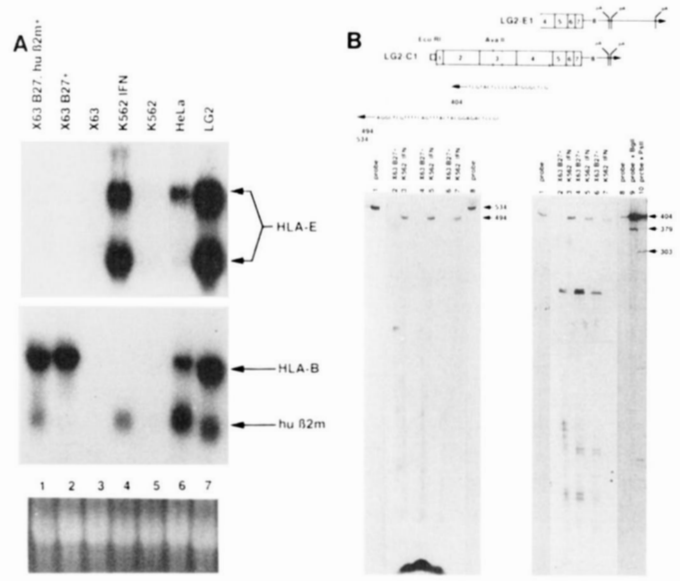

Figure 5. *A*, Characterization of the RNA preparations used in the S1 nuclease protection assays. A total of 15 μg of total RNA isolated from HLA-B27/human β_2m$^+$X63 transfectants (*lane 1*); HLA-B27$^+$ X63 transfectants (*lane 2*); untransfected X63 cells (*lane 3*); IFN-γ- stimulated (*lane 4*) and unstimulated K562 cells (*lane 5*); HeLa cells (*lane 6*); B27 homozygous B cell line LG2 (*lane 7*) were tested by Northern blot analysis for the expression of the HLA-E gene (*top*, hybridization with oLG2-C1), the expression of the HLA-B locus and human β_2m (*middle*, hybridization with the B specific and a human β_2m probe). The amount of RNA loaded was controlled by ethidium bromide staining of the 18 S rRNA (*bottom*). *B*, S1 nuclease protection assays. *Upper panel*, The origin of the continuously labeled overlapping single-stranded antisense probes is shown. The location and sequence of the primers and the restriction sites used in the PCR generation of the probes with the cDNA LG2-C1 in pUC18 as template are represented. Note that the 404 nucleotide exon 3/4 fragment overlaps with the sequence of the LG2-E1 cDNA. *Lower panel, left*: The 534 nucleotide probe covering exon 1 to 3 and 40 nucleotides of the pUC18 polylinker (boxed in *upper panel*) (*lanes 1* and *8*) was hybridized to total RNA from either the HLA-B27$^+$ X63 transfectant (*lanes 2*, 4, and 6) or K562 stimulated with 1000 U/ml IFN-γ for 48 h (*lanes 3*, 5, and 7), digested with 600 U (*lanes 2* and *3*), 1200 U (*lanes 4* and *5*), or 2400 U (*lanes 6* and 7) of S1 nuclease at 32°C for 2 h, run on a 4% acrylamide/7 M urea gel, and autoradiographed. *Lower panel, right*: The 404 nucleotide probe representing exon 3 and 4 (*lanes 1* and *8*) was hybridized to total RNA from either the B27$^+$ X63 transfectant (*lanes 2*, 4, and *6*) or stimulated (see above) K562 (*lanes 3*, 5, and 7), digested with 600 U (*lanes 2* and 3), 1200 U (*lanes* 4 and 5), or 2400 U (*lanes 6* and *7*) of S1 nuclease at 32°C for 2 h, run on a 4% acrylamide/7 M urea gel, and autoradiographed. The bands derived from B27$^+$X63 cells (*lanes 2*, 4, and *6*) represent most likely protected sequences of exon 4 that are conserved between HLA-B27 and HLA-E. Additional size standards were generated by restriction of the probe after PCR amplification with *Bgl*II (*lane 9*) or *Pst*I (*lane 10*). Note that due to PCR generation only 10% of the probe is double-stranded DNA and can be cleaved by the restriction enzymes.

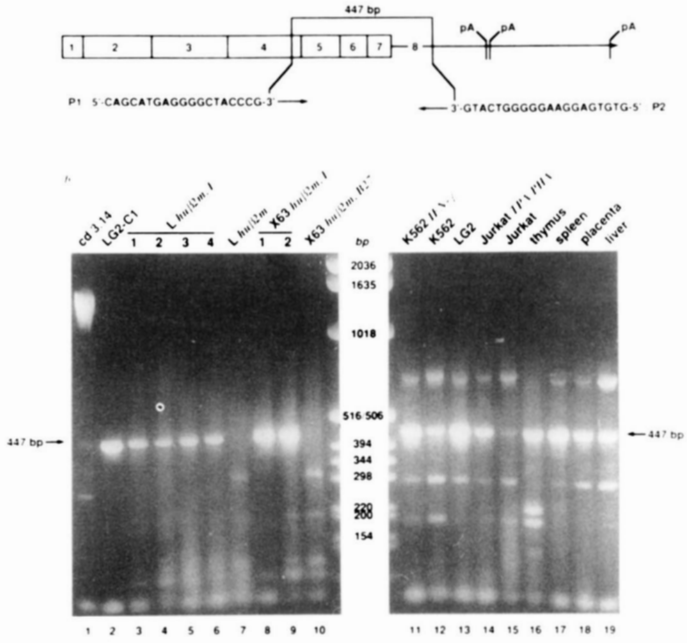

Figure 6. PCR amplification of a region covering the 3'end of exon 4 to the 3' UT of the HLA-E mRNA. *a*, Location and sequence of the primers P1 and P2 used for amplification of the 3' portion of the HLA-E mRNA. *b*, 2 μg of total RNA from the indicated cells were used for first-strand cDNA synthesis. A total of 50 cycles of PCR amplification was carried out with the P1 and P2 primers on 1/30 of the cDNA reaction mix prepared from pLG2-C1/KSVneo (*lane 3*) and three independent cd3.14 (*lanes 4–6*) transfected human β_2m$^+$ L cells, untransfected human β_2m$^+$ L cells (*lane 7*), two independent cd3.14/human β_2m transfeceted X63 clones (*lanes 8* and 9), a human β_2m/B27$^+$ X63 transfectant clone (*lane 10*), resting (*lane 12*), and IFN-γ-stimulated (*lane 11*) K562 cells, unstimulated LG2 cells (*lane 13*), untreated (*lane 15*), and 12-*O*-tetradecanoylphorbol 13-acetate/PHA-stimulated (*lane 14*) Jurkat cells and the following human tissues: thymus (*lane 16*), spleen (*lane 17*), placenta (*lane 18*) and liver (*lane 19*). Specificity of the 447-bp amplification product coding for a 3' unspliced HLA-E mRNA segment was confirmed by hybridization with an oligomer specific for the transmembrane region (5'-AGA GAC CAC AGA TCC AAG GAG A-3') of HLA-E mRNA (data not shown). One pg of either cd3.14 (*lane 1*) or LG2-C1 (*lane 2*) were amplified to exclude contamination with DNA and demonstrate specificity, respectively.

No HLA α-chains could be immunoprecipitated from culture supernatants.

DISCUSSION

Previous reports on the transcription of the HLA-E gene used probes from the 3' untranslated region of the HLA-E gene that either cross-react with HLA-A, -B, and -C transcripts (4, 5, 12) or hybridize exclusively to the large transcript (4, 31, 12). We used an oligonucleotide that is strictly HLA-E specific and detects both transcripts, and could show that the ratio between the two messages is variable in different tissues and is related to cell activation in vitro.

The differential and tissue-specific regulation of the two HLA-E transcripts led us to determine the nature of the 2.7-kb mRNA. cDNA sequence analysis, S1 nuclease protection assays and PCR cDNA amplification showed that both transcripts have identical protein coding sequences and differ only in the extent of the 3' untranslated region. The differential expression of the two HLA-E mRNA species is controlled by alternative polyadenylation. The use of alternative polyadenylation signals in the 3' untranslated region has also been described for transcripts of *Tla* genes in the mouse (32, 33), but its significance with regard to thymus leukemia Ag expression is not known. Moreover, the mechanisms controlling alternative poly(A) site usage are also not yet well understood (34). Interestingly, only recently a role of the 3' untranslated region in the control of translation efficiency has become apparent (30). It has been shown that downstream elements in cytokine transcripts are capable of suppressing their translation (35, 36).

Conflicting results concerning HLA-E cell surface expression have been reported (3, 8), and the possibility remained that the lack of surface expression of the HLA-E H chain upon transfection into the human B-LCL.221 might be the result of the mutant phenotype of this cell line, or due to the transfection of the *Hin*dIII subclone that only encodes the short 1.8-kb HLA-E transcript (3). Initially, we transfected both an eucaryotic expression construct of the HLA-E cDNA LG2-C1, which should only result in the synthesis of the 1.8-kb HLA-E transcript, and the cosmid cd3.14 that also codes for the 2.7-kb

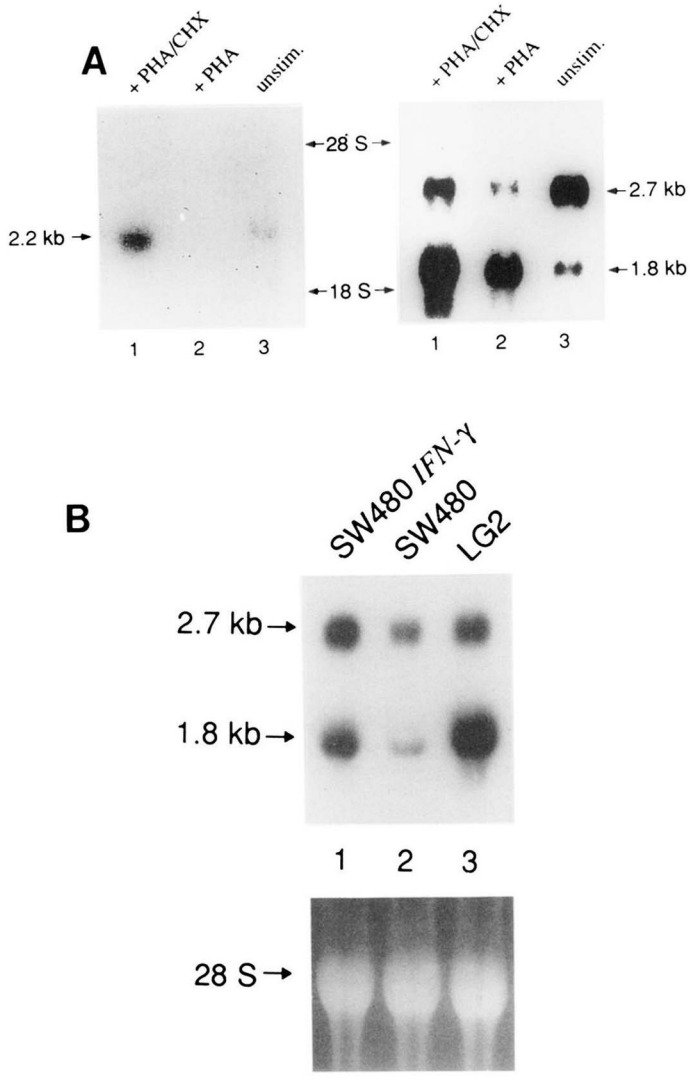

Figure 7. *A*, Induction and stability of HLA-E mRNA in PBL. A total of 10 μg of total RNA per lane isolated from either resting PBL or PBL after PHA or PHA/CHX treatment, respectively, was fractionated on a formaldehyde agarose gel and hybridized either with the c-fos-specific 60 mer c-fos/o1 (*left*) or with the 33 mer oLG2-C1 (*right*). *B*, Differential induction of both HLA-E transcripts in SW480 by IFN-γ. A total of 10 μg of total RNA per lane isolated from unstimulated (*lane 2*) or IFN-γ-treated (*lane 1*) SW480 cells and untreated LG2 cells (*lane 3*), was separated on a formaldehyde agarose gel and hybridized with oLG2-C1. The amount of RNA loaded was controlled by ethidium bromide staining of the 28 S rRNA (*bottom*).

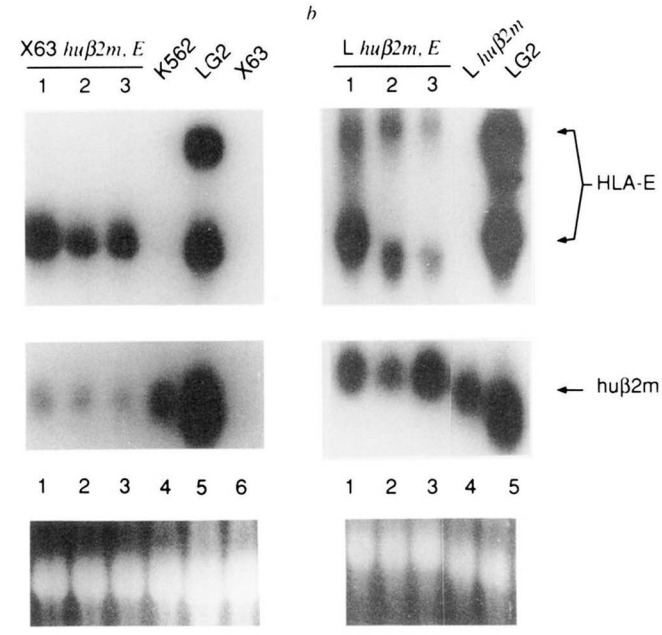

Figure 8. Detection of HLA-E and human β_2m mRNA in human β_2m^+ L cell (*a*) and X63 (*b*) transfectants. *a*, 15 μg of total RNA from three representative human β_2m/E^+ X63 transfectant clones (*lanes 1–3*), unstimulated K562 (*lane 4*), LG2 (*lane 5*), and untransfected X63 (*lane 6*) were separated on a formaldehyde agarose gel and probed with either oLG2-C1 (*top*) or the human β_2m probe (*middle*). Exclusively, the 1.8-kb HLA-E transcript is present in the transfectants (*lanes 1–3*). All transfectants transcribe human β_2m. Identity of the amounts of RNA loaded was controlled by ethidium bromide staining of the 18 S rRNA (*bottom*). *b*, 15 μg of total RNA from three independent cd3.14- transfected human β_2m^+ L cell clones (*lanes 1–3*), untransfected human β_2m^+ L cells (*lanes 4* and *5*), and LG2 cells (*lane 6*) were separated on a formaldehyde agarose gel and probed either with oLG2-C1 (*top*) or the human β_2m probe (*middle*). Human β_2m/E^+ L cells transcribe mainly the 1.8-kb HLA-E mRNA. A 2.7-kb signal is visible due to overexposure as can be estimated from the intensity of the HLA-E signals in LG2 (*lane 6*). The amount of RNA loaded was controlled by ethidium bromide staining of the 18 S rRNA (*bottom*).

transcript, into mouse cell lines. Inasmuch as on transfection of the cosmid the 1.8-kb mRNA is mainly synthesized, only it was used in later experiments. Cell surface expression was only observed after transfection of the mouse myeloma cell line X63. This result might reflect genetic variations in MHC encoded transporter and/or proteasome genes that by controlling the endoplasmic peptide spectrum influence class I assembly and thus cell surface expression (37–39). Finally, we cannot exclude that the failure to detect the HLA-E Ag in J27 and P815 transfectants might be due to the broader staining and higher nonspecific antibody binding profiles of untransfected cells compared to X63 cells (Fig. 8) that could obscure a weak HLA-E signal.

Recently, the absence of cell surface expression of Qa-2 Ag in L cells transfected with the Q7 and Q9 genes, was explained by the predominant synthesis of alternatively spliced mRNA lacking exon 5 (40). Therefore, we also asked whether a similar mechanism might lead to the secretion of the HLA-E molecule. PCR amplification demonstrates that all transfectants express the same HLA-E mRNA, containing exon 5 (Fig. 6), and confirm our previous finding that all HLA-E transcripts code for the same H chain. Moreover, no HLA class I H chain was detected in culture supernatants of the transfectants.

The absence or low levels of intracellular and cell surface expression of the HLA-E H chains in all transfectant clones, irrespective of the amount of HLA-E mRNA present, might be explained by a low translation efficiency and/or inadequate assembly and transport of the HLA-E molecule in the mouse cell lines. Accumulating evidence suggests that the synthesis of a class I polypeptide is not the only requirement for cell surface expression of the class I transplantation Ag, but that additional factors are necessary which control processing and intracellular trafficking in a cell type and locus/allele-specific manner (37, 38, 41–43). It is conceivable that the same or similar mechanisms regulate cell surface transport of HLA-E molecules. The inefficient cell surface transport is not likely to be due to the HLA-E leader peptide, because the cDNA construct pLG2-C1/KSVneo that codes for an HLA-B8 leader peptide is not expressed at the cell surface. The cell surface expression of the HLA-E Ag in the X63 transfectants indicates that the HLA-E Ag might be a cell surface molecule in vivo. After transfection of the HLA-E gene into mouse cell lines, the 1.8-kb transcript is

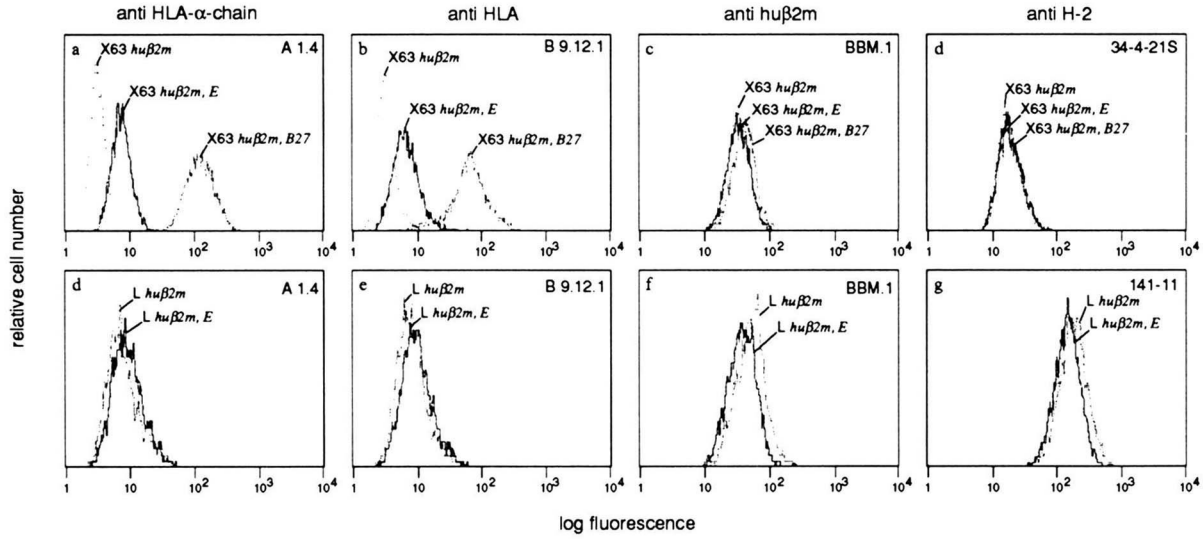

Figure 9. Binding of the mAb directed against HLA class I α-chains (A1.4), HLA class I complexes (B9.12.1), H-2D^d (34-4-21S), H-2K^k (141-11), and human β_2m (BBM.1) to X63 and human β_2m$^+$ L cell (J27) transfectants. Each diagram of the *upper row* (*a-d*) compares the binding of the indicated antibody to human β_2m$^+$, human β_2m/HLA-E$^+$, and human β_2m/HLA-B27$^+$ X63 transfectants. In the *lower row* each diagram (*e-g*) compares the staining with the indicated antibody of the human β_2m- expressing L cell to that of its HLA-E transfectant. For X63 cells mAb 34-4-21S was used to determine H-2D^d expression. In the case of the L cell transfectants, H2-K^k expression was assessed with mAb 141-113.

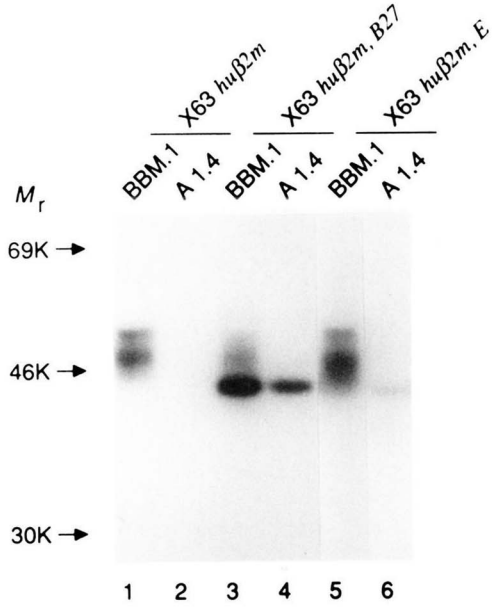

Figure 10. Immunoprecipitation of iodinated cell surface HLA class I H chains. Lysates of labeled human β_2m$^+$ (*lanes 1* and *2*), human β_2m/B27$^+$ (*lanes 3* and *4*), and human β_2m/E$^+$ (*lanes 5* and *6*) X63 transfectants were sequentially immunoprecipitated with mAb: isotype control, anti- human CD4, M-T242; anti-human class I H chain, A1.4 (*lanes 2, 4,* and *6*), and finally anti-human β_2m, BBM.1 (*lanes 1, 3,* and *5*). Analysis was performed by 10% SDS-PAGE (*lanes 1-4* were exposed for 7 days and *lanes 5* and *6* for 10 days). BBM.1 also precipitates mouse class I molecules complexed with human β_2m from all X63 transfectants (*lanes 1, 3,* and *5*). A faint HLA-E-derived α-chain is visible below the H-2 class I H chains ih the BBM.1 immunoprecipitate (*lane 5*). The presence of human class I α-chains in the transfectants reduced complex formation of human β_2m with endogenous H-2 class I H chains.

mainly synthesized and only a low amount of intracellular and cell surface HLA-E class I molecule is detected. Inasmuch as differential regulation of the two transcripts was demonstrated in vivo, the possibility remains that only the 2.7-kb HLA-E transcript leads to efficient translation and cell surface expression of HLA-E Ag due to genetic elements responsible for posttranscriptional control encoded by its extended 3′ untranslated region.

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