Exon 5 Encoding the Transmembrane Region of HLA-A Contains a Transitional Region for the Induction of Nonsense-Mediated mRNA Decay

Yumiko Watanabe, Katharine E. Magor and Peter Parham


http://www.jimmunol.org/content/167/12/6901

---

**References**

This article cites 77 articles, 32 of which you can access for free at:
http://www.jimmunol.org/content/167/12/6901.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Exon 5 Encoding the Transmembrane Region of HLA-A Contains a Transitional Region for the Induction of Nonsense-Mediated mRNA Decay

Yumiko Watanabe, Katharine E. Magor, and Peter Parham

HLA class I alleles containing premature termination codons (PTCs) are increasingly being found. To understand their effects on MHC class I expression, HLA-A*2402 mutants containing PTCs were transfected into class I-deficient cells, and expression of HLA-A mRNA and protein was determined. In exons 2, 3, and 4, and in the 5' part of exon 5, PTCs reduced mRNA levels by up to 90%, whereas in the 3' part of exon 5 and in exons 6 and 7 they had little effect. Transition in the extent of nonsense-mediated mRNA decay occurred within a 48-nt segment of exon 5, placed 58 nt upstream from the exon 5/exon 6 junction. This transition did not conform to the positional rule obeyed by other genes, which predicted it to be ~50–55 nt upstream of the exon 7/exon 8 junction and thus placing it in exon 6. Mutants containing extra gene segments showed the difference is caused by the small size of exons 5 and 6, which renders them invisible to the surveillance machinery. For the protein, a transition from secretion to membrane association occurs within a 26-nt segment of exon 5, 17 nt upstream of the exon 5/exon 6 junction. Premature termination in exon 5 can produce secreted and membrane-associated HLA-A variants expressed at high levels. The Journal of Immunology, 2001, 167: 6901–6911.

P

letic mutation in the expression of HLA-A class I alleles. HLA-A, B, and C, has been extensively studied because of its medical importance in transplantation (1, 2). Recently, introduction of DNA methods of HLA typing facilitated the identification and characterization of a type of HLA class I allele not readily detected by the serological methods used in clinical HLA analysis. These null alleles have substitutions, insertions, or deletions that prevent or reduce the expression of an HLA class I molecule at the cell surface. To date some 25 HLA class I null alleles have been described (3–21). HLA class II null alleles are also known, but to a lesser extent than for class I (22–29).

In general, a null allele is very closely related in sequence to a normally expressed allele from which it has likely evolved. A majority of HLA class I null alleles are inactivated by the presence of a premature termination codon (PTC). These can be caused directly by point substitution within a codon, or indirectly by nucleotide insertion or deletion that changes the reading frame and leads to premature termination downstream. Null alleles are characterized by low or undetectable levels of mRNA (10–12) that limit the production of truncated class I H chain protein. Studies on other eukaryotic genes have revealed a pathway called nonsense-mediated mRNA decay (NMD) in which PTCs trigger the degradation of the mRNA containing them (30–35). The mechanism is yet to be defined precisely, but it is best understood in yeast (31, 33).

In yeast, NMD is initiated during translation of the message in the cytoplasm. PTCs are distinguished from the normal termination codon by the presence of specific sequence elements downstream of the PTC. Such downstream sequence elements can occur at several places within the coding region and can activate NMD when situated within ~150 nt 3' of a PTC. Also found in yeast genes are sequence elements termed stabilizer elements that can inactivate NMD (36). Initiation of NMD is coupled to translation termination at a PTC. A current model postulates that NMD initiates when the first ribosome to translate a message fails to dissociate specific proteins downstream of the PTC, proteins that were bound to the downstream sequence elements in the nucleus and retained on delivery to the cytoplasm. The retention of such proteins in complex with mRNA (in PTC-containing, but not normal, transcripts) is then believed to stimulate a series of reactions that lead to removal of the 5' cap and degradation of the message by a 5'-3' exonuclease (31, 33).

PTCs in several mammalian genes have been shown to reduce steady state levels of mRNA (34). These genes include those encoding dihydrofolate reductase (DHFR) (37), adenine phosphoribosyltransferase (38), β-globin (39–41), triosephosphate isomerase (TPI) (42–47), major urinary protein (MUP) (48), TCR (49, 50), and Ig (51). A major difference between NMD in mammals and yeast is the involvement of introns in mammalian genes. In addition to their effect on mRNA level, PTCs in mammalian genes can affect mRNA splicing, causing exon skipping (52) and/or intron retention (53). Such phenomena have suggested that specific recognition of mammalian PTCs takes place in the nucleus, a view.

Copyright © 2001 by The American Association of Immunologists

0022-1767/01/$02.00
first espoused in the nuclear scanning model of NMD of Urlaub et al. (37). In contrast, factors affecting translation also influence NMD (for example, suppressor tRNAs and mutated start codons), implicating translation as the process during which PTCs are identified and the NMD pathway is initiated (46, 50, 53, 54). Recent report of the coupling of transcription and translation in discrete transcriptional “factory” sites within mammalian nuclei might explain why both nuclear and cytoplasmic associated NMD have been observed in eukaryotes (55).

Initiation of mammalian NMD requires both a PTC and a downstream element that are contributed from the removal of an intron while inside the nucleus (42–44, 49). The cotranslational model of Maquat (56) and the posttermination model of Hentze and Kulozik (32) both propose that PTC surveillance requires machineries involved in protein translation. In these models, the template for surveillance is fully spliced mRNA that has acquired marks or tags deposited at the exon/exon junctions after the splicing out of introns (32, 39, 42, 49, 57). Such markers could be proteins (e.g., Upf3) that were acquired in the nucleus and that in the first translation of a normal message are dissociated from the mRNA by translocation of the ribosome (58). Premature termination would not dissociate the proteins from downstream junctions, and their persistence as complexes with the mRNA could induce the NMD pathway.

From the studies on MUP, TPI, and β-globin, it has been shown that one downstream intron is necessary for a PTC to trigger the NMD pathway, and the PTC needs to be at least 50–55 nt upstream of the junction of the last two exons (the two 3’-most exons) in the fully spliced message (39, 40, 42). Nagy and Maquat (30) have generalized this positional rule to explain why the normal termination codon is within the last exon of most genes, or, in a minority of genes (7% of 1500 surveyed), within a region 50–55 bp upstream from the 3’-most exon/exon junction: termination codons upstream of this region would lead to transcript loss through NMD (30). Zhang et al. (42) hypothesize that 50–55 nt is the minimum distance necessary for the NMD-scanning mechanism to recognize both the termination codon and the marker at the downstream exon/exon junction. Current candidates for the scanner involve the 40S ribosomal complex or ribosomal termination complex, possibly in association with the Upf1 protein (59), which interacts with translation termination factors (60, 61). It is proposed that the Upf3 protein first associates with the exon/exon junction of a transcript in the nucleus upon intron splicing. Upf3 then binds with the Upf2 protein to form a complex, which then interacts with Upf1 via the translation release factors on the ribosome and induces NMD (58, 61).

A characteristic of gene families encoding Ag-recognition molecules of immunity is rapid evolution through mutational processes that inevitably generate PTCs. During T and B cell development, PTCs are introduced at high frequency by the gene rearrangements and accompanying reactions that are used to make functional Ig and TCR. Expression of these PTC-containing genes is down-regulated by the NMD pathway (49–51, 62). MHC class I and II genes are the most polymorphic mammalian genes known, their diversity and rapid evolution representing host responses to the selection imposed by pathogens. Because MHC molecules select the T cell repertoire as well as present Ags to T cells, we have previously proposed that pathogens, depending on circumstance, can select for loss of function and expression in MHC alleles as well as for presentation of novel Ags (63). In this regard, it is intriguing that one of only two novel genes that Nagy and Maquat (30) found not to obey the 50- to 55-nucleotide rule was the nonclassical class I gene HLA-G. To more fully understand the effects that naturally occurring PTCs have on the expression of MHC class I genes, we have performed an analysis of PTC-containing mutants of a common human MHC class I allele (HLA-A*2402).

Materials and Methods

Cells

EBV-transformed human B-lymphoblastoid cell lines (B-LCL) were cultured in Cellgro RPMI 1640 (Mediatech, Herndon, VA) supplemented with 10% bovine calf serum (BCS). The B-LCL JY expresses HLA-A*0201 and HLA-B*0702, and TISI expresses HLA-A*2402 and HLA-B*3508. The HLA class I-deficient cell line, 721.221, was used as the recipient for HLA class I genes in transfection experiments (65).

Antibodies

The HLA class I-specific mAb, W6/32 (IgG2a), recognizes a monomorphic epitope on the class I H chain and β2m complex (66). Rabbit polyclonal antisera, ABR2, was raised against a peptide (CAQGSDVSLTA) corresponding to residues 330–339 of the cytoplasmic tail of HLA class I H chain (67). HRP-conjugated rabbit anti-human β2m Ab PA174 was purchased from DAKO (Glostrup, Denmark).

DNA mutagenesis and plasmid constructs

Nonsense mutations were introduced into the HLA-A*2402 gene using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), as instructed by the manufacturer. DNA sequences of mutated fragments and PCR products were analyzed using an ABI 373A DNA sequencer (Applied Biosystems, Foster City, CA). The nucleotide sequence of HLA-A*2402 was determined by Magor et al. (6) (GenBank accession number L47206); it contains the entire ~5-kb HindIII genomic fragment including the promoter, 5’-untranslated region, exons 1–8 and intervening introns, and the 3’-untranslated region. The numbering used in this study starts with the first nucleotide of the submitted sequence of 6705 nt.

Mutants M2:53, M2(TGA):53, M3:99, M3(TAA):99, and M3(TGA):99. A 1.33-kb Eagl-Ndel fragment, corresponding to nt 1438 (exon 2) to 2775 (exon 4), was excised from the A*2402 gene and subcloned into the pGEM 5ZI vector (Promega, Madison, WI). Nonsense mutations in exons 2 or 3 were created by site-directed mutagenesis with primer pairs listed below. The mutated fragment was excised with PstI and XhoI, and replaced the normal PstI-XhoI fragment, corresponding to nt 1519 (exon 2) to 2695 (intron 3), in the A*2402 gene that was previously subcloned in pBlueScript KS+ (Stratagene). The ~5-kb HindIII fragment containing the entire mutant A*2402 gene was then transferred from pBlueScript KS+ into the pHeBo expression vector (68, 69). The following oligonucleotide primer pairs were used for mutagenesis, and the mutations of interest were underlined: for M4:257, 5’-GAGGAGCAAGATGGTCATCT-3’; for M5:272, 5’-ACCTTCAACCATACCTCTGAGG-3’; for M6:275, 5’-CTCTTACACCATACCTCTGAGG-3’; and for M7(TGA):280, 5’-ACCTTCAACCATACCTCTGAGG-3’.

Mutants M4:257, M4(TGA):257, M4:262, M4:274, M4:275, M5:276, M5:279, M5:295, M5:300, M5:308, M6:315, M7:330, and M7(TGA):339. A 1.32-kb SphI fragment, corresponding to nt 2697 (exon 3) to 4013 (intron 7), was excised from the A*2402 genomic DNA and subcloned into the pGEM 5ZI vector. Nonsense mutations in exons 5, 6, and 7 in pGEM ZI (SphI) were created by site-directed mutagenesis with primer pairs listed below. Mutated fragments were excised with SphI and replaced the normal SphI fragment (corresponding to nt 2697–4013) in the A*2402 gene in pBlueScript SK+. The ~5-kb HindIII fragment containing the entire mutant A*2402 gene was then transferred into the pHeBo expression vector (68, 69). The following oligonucleotide primer pairs were used for mutagenesis, and the mutations of interest were underlined: for M4:257, 5’-GAGGAGCAAGATGGTCATCT-3’; for M5:272, 5’-ACCTTCAACCATACCTCTGAGG-3’; for M6:275, 5’-CTCTTACACCATACCTCTGAGG-3’; and for M7(TGA):280, 5’-ACCTTCAACCATACCTCTGAGG-3’.

Mutants M4:257, M4(TGA):257, M4:262, M4:274, M4:275, M5:276, M5:279, M5:295, M5:300, M5:308, M6:315, M7:330, and M7(TGA):339. A 1.32-kb SphI fragment, corresponding to nt 2697 (exon 3) to 4013 (intron 7), was excised from the A*2402 genomic DNA and subcloned into the pGEM 5ZI vector. Nonsense mutations in exons 5, 6, and 7 in pGEM ZI (SphI) were created by site-directed mutagenesis with primer pairs described below. Mutated fragments were excised with SphI and replaced the normal SphI fragment (corresponding to nt 2697–4013) in the A*2402 gene in pBlueScript SK+. The ~5-kb HindIII fragment containing the entire mutant A*2402 gene was then transferred into the pHeBo expression vector. Primer pairs used for mutagenesis were as follows: the positions of mutation in the sequences are underlined: for M4:257, 5’-GAGGAGCAAGATGGTCATCT-3’; for M5:272, 5’-ACCTTCAACCATACCTCTGAGG-3’; for M6:275, 5’-CTCTTACACCATACCTCTGAGG-3’; and for M7(TGA):280, 5’-ACCTTCAACCATACCTCTGAGG-3’.
and 5′-GGGCTGGGAGATATCATCGGGAAAAG-3′; for M5:279, 5′-GAGCCATCTTCTGAGCCCGCTCC-3′ and 5′-GGGAGGGTTGGGTATGGCAG-3′; for M5:290, 5′-GGCTGCTTCTCTTTGAGCT-3′ and 5′-GGCTGCTTCTCTTTGAGCT-3′; for M5:300, 5′-GCTGTGATCATTGCTTGTCGC-3′ and 5′-GACGCCAGCCTGACATGTCAG-3′; for M5:308, 5′-GCTGTGATCATTGCTTGTCGC-3′ and 5′-GACGCCAGCCTGACATGTCAG-3′; for M5:321, 5′-GACATCGAGCCCTGACCTGACT-3′ and M3:151, 5′-GACATCGAGCCCTGACCTGACT-3′; for M3:210, 5′-GACATCGAGCCCTGACCTGACT-3′ and M3:270, 5′-GACATCGAGCCCTGACCTGACT-3′. Plasmid constructions containing extra exon or intron fragments were inserted into the BglII site at position 3515 in intron 5 of A*2402 and M5:308. A PCR was made that contained plasmid template (50 ng), dNTPs (2 mM each), two primers (25 pmol), and 2.5 U native Pfu DNA polymerase in 50 µl 1× reaction buffer (Stratagene). Amplification of the DNA fragment was performed for 28 cycles of 20 s at 94°C, 25 s at 60°C, and 90 s at 72°C.

**Mutants E4, E4:257, E4:262, and E4:274.** DNA fragments containing normal or mutated exon 4 were amplified by PCR using the sense oligonucleotide Bgl4F 5′-GCGAGATCTGTAGCAGATGCAAAATGCCTGAA-3′ (the T7 promoter sequence is underlined) and the antisense oligonucleotide Bgl4R 5′-GGGCTGGGAAGATTACTCTGGGAAAAGAG-3′ (nucleotide corresponding to 3515) in intron 5 of the A*2402 gene. DNA fragments containing normal exon 6 and 7 were amplified from A*2402 by PCR using sense oligonucleotide Bgl6F 5′-GGCGAGATCCTGTGACTACCCGACCCGGGCTC-3′ and antisense oligonucleotide Bgl6R 5′-GGCGAGATCTGTAGCAGATGCAAAATGCCTGAA-3′ (BglII restriction sites are underlined). DNA fragments containing normal exons 6 and 7 were amplified from A*2402 by PCR using sense oligonucleotide Bgl6F 5′-GGCGAGATCCTGTGACTACCCGACCCGGGCTC-3′ and antisense oligonucleotide Bgl6R 5′-GGCGAGATCTGTAGCAGATGCAAAATGCCTGAA-3′ (BglII restriction sites are underlined). DNA fragments containing normal exon 6 were amplified from A*2402 by PCR using sense oligonucleotide Bgl6F 5′-GGCGAGATCCTGTGACTACCCGACCCGGGCTC-3′ and antisense oligonucleotide Bgl6R 5′-GGCGAGATCTGTAGCAGATGCAAAATGCCTGAA-3′ (BglII restriction sites are underlined). DNA fragments containing normal exon 6 and 7 were amplified from A*2402 by PCR using a primer Bgl3R 5′-CACATCAGAGCCCTGACCTGACT-3′ (underlined) from template plasmids A*2402, M4:257, M4:262, and M4:274. The PCR products were digested with BglII and inserted into the BglII site in intron 5 of the A*2402 gene (situated at nt 3515).

**Transfection.** The HLA-A, B, C-deficient B-LCL 721.221 cells (1.25 × 10⁶) were transduced by electroporation with 20 µg of either the wild-type HLA-A*2402 gene or mutant constructs cloned into the pHeBo vector, as previously described (71). Transfectants were cultured in the presence of 0.3% DMSO (Sigma-Aldrich, St. Louis, MO) and 5 µg/ml hygromycin (Calbiochem, San Diego, CA) to ensure retention of the episomal replicating pHeBo vector.

**Isolation of RNA.** Total RNA was isolated from transfected cells and control 721.221 cells using the TRI reagent (Molecular Research Center, Cincinnati, OH), as recommended by the manufacturers. Cell fractionation was performed as described (71), with minor modification. Cells were washed twice with PBS and resuspended in hypotonic buffer (10 mM Tris (pH 7.4), 2 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, and 1 mM DTT) containing 1,000 U/ml RNasin (Promega). The mild detergent Nonidet P-40 was added to the cell suspension (final concentration 0.3%) and incubated for 5 min. After being passed through a 22-gauge needle, nuclei were pelleted by centrifugation at 1,000 rpm for 5 min. The supernatant was further centrifuged at 14,000 rpm, and the second supernatant fraction was used as a cytoplasmic fraction. Ten volumes of TRI reagent were added to the cytoplasmic fraction, and cytoplasmic RNA was prepared as described above. Nuclei were resuspended with the hypotonic buffer without DTT and RNasin, and were purified by centrifugation through a 0.25 M sucrose cushion at 1,000 rpm. Nuclear RNA was isolated using the TRI reagent, as described above.

**Northern blot analysis.** Eight micrograms of total, nuclear, or cytoplasmic RNA were used for analysis. Northern blotting was performed using Northern Max kit (Ambion, Austin, TX) according to the manufacturer’s instructions. RNA probes were generated by PCR amplification, followed by in vitro transcription using the Strip-EZ RNA kit (Ambion). Primers for PCR amplification were as follows: for HLA-A, sense oligonucleotide 5′-TTTGTGTTGGACTGAGGCGAAGTGT-3′ and antisense oligonucleotide 5′-TAA TACACTCTTATAGGGAAAAGCCTGGGAGAAGC-3′ (the 17 promoter sequence is underlined); for β2m, sense oligonucleotide 5′-GAAGGAAATGGGCACTTACAGG-3′ and antisense oligonucleotide 5′-TTATACGACTCTATAGGGCCTATAC CTTCTTGAGATGTGGTTC-3′; and for the Escherichia coli hygromycin resistance gene (hyg), which is part of the pHeBo vector, sense oligonucleotide 5′-GATTCGGAAGTCTGACATATTGTT-3′ and antisense oligonucleotide 5′-TTAACGACTCTATAGGGCCTATAC CTTCTTGAGATGTGGTTC-3′.

**Flow cytometry.** Approximately 5 × 10⁵ cells were incubated with 2 µg of purified W6/32 Ab (BioSource, Camarillo, CA). Cell surface expression of HLA class I molecules was measured using a FACScan flow cytometer (BD Biosciences, San Jose, CA). Dead cells were excluded from the analysis by propidium iodide staining.

**Enzyme-linked immunosorbent assay.** Cells (5 × 10⁵/ml) were cultured either in RPMI 1640/10% BCS (for 72/221) or in RPMI 1640–30% BCS containing 400 µg/ml hygromycin (for transfectants). Cell culture supernatants were collected after 1 day and filtered with a 0.22-µm filter to remove residual cell debris. HLA class I molecules were detected using a sandwich ELISA system, as previously described (71). Briefly, Maxisorp Immunoplates (Nunc, Naperville, IL) were coated with 100 µl of W6/32 Ab (2 mg/ml in PBS) overnight at 4°C. Plates were blocked with 2% BSA in PBS for 30 min at 20°C. Cell culture supernatants (1/25, 1/50, 1/100 dilutions) were added to the blocked plates. Plates were incubated for 2 h at room temperature. One hundred microliters of HRP-conjugated anti-human β2m Ab (1/2000 dilution) were added, and the plates were incubated for 2 h at room temperature. Two hundred microliters of o-phenylenediamine peroxidase substrate solution (Sigma-Aldrich, St. Louis, MO) were added to each well. After a 30-min incubation, the color reaction was stopped with 3 N hydrochloric acid, and absorbance at 490 nm was measured using a microtiter plate reader.

**Metabolic radiolabeling.** Cells (1 × 10⁵/ml) were preincubated for 1 h in methionine- and cysteine-free RPMI 1640 medium (Life Technologies, Rockville, MD) containing 10% dialyzed FCS (Sigma-Aldrich) at 37°C (10-35S)methionine (70 µCi) (SJ1015; Amersham Pharmacia Biotech, Piscataway, NJ) was added, and cells were incubated for 5 h.

**Immunoprecipitation and isoelectric focusing gel electrophoresis.** Lysis and immunoprecipitation were performed as described previously (72). Briefly, cell lysates were precleared with normal mouse IgG or normal rabbit serum, and Staphylococcus aureus aureus cells (Roche Molecular Biochemicals, Indianapolis, IN) to reduce nonspecific binding. Precleared lysates were incubated in the presence of 5 µg of mouse mAb W6/32 or 5 µl of AB R2 rabbit antisera for 1 h at 4°C. Immunoprecipitation was performed in the presence of 50 µl of S. aureus cells. Immune complexes were washed, treated with 20 µl of neuraminidase type VIII (Sigma-Aldrich), and analyzed by isoelectric focusing (IEF). Banding patterns were visualized by autoradiography.

**Results.** The purpose of this investigation was to assess the effects of PTCs when introduced at different sites within an allele of a highly polymorphic, classical HLA class I gene. To study this question, we generated mutants of HLA-A*2402, an allele that is prevalent in many human populations and one for which we have previously described natural variants containing PTCs (11).
FIGURE 1. PTCs in exons encoding the extracellular domains of the HLA-A*2402 H chain gene cause down-regulation of mRNA and cell surface protein expression. A, Shows the exon-intron organization of the normal HLA-A*2402 gene and of the mutant constructs containing PTCs in exons 2–7. Exons containing a PTC are stippled. HLA class I-deficient 721.221 cells were transfected with wild-type and mutant constructs in the pHeBo vector, and Northern blotting analysis was performed on total RNA (B), nuclear RNA (C, left), and cytoplasmic RNA (C, right). The blots were sequentially probed for the bacterial hyg' of the pHeBo vector, for HLA-A, and for β2-m. Although lacking functional HLA-A gene, the 721.221 cell line has normal β2m gene. As controls, untransfected 721.221 cells and 721.221 cells transfected with vector alone were included in the blotting analysis. HLA-A mRNA levels are expressed as a percentage of normal A*2402 mRNA after normalization to the level of hyg' mRNA. The diamond (♦) indicates signal on the HLA-A blot that remained from previous hyg' hybridization and that was not completely removed during dehybridization. D, Transfectants expressing the
PTCs in exons 2, 3, and 4, but not exons 5, 6, and 7, reduce levels of HLA-A mRNA

PTCs were individually introduced into exons 2–7 of the HLA-A*2402 gene. Together these six exons specify the 341 residues of the mature HLA-A*2402 protein (Fig. 1A). Wherever possible, mutations were chosen to mimic ones present in the human population either in expressed or null HLA class I genes. Thus, the mutation made in exon 3 (Phe29 to stop) corresponds to that in HLA-A*1526N (12), the mutation made in exon 4 (Tyr295 to stop) corresponds to that in A*0215N (5), and the mutation made in exon 6 (Arg311 to stop) corresponds to the normal site of termination in the oligomeric nonclassical class I gene, HLA-G (64).

Genomic DNA fragments containing the A*2402 promoter/enhancer sequence and either normal or mutated A*2402 genes were subcloned into the pHeBo expression vector containing a hygromycin resistance gene. HLA-A, B, C-deficient 721.221 cells were transfected with the normal or mutated A*2402 genes, and stable cell lines were established by culture with hygromycin. Northern blot analysis of total RNA revealed that the HLA-A mRNA levels were decreased in transfecteds expressing mutants having a PTC in exon 2, 3, or 4 (M2:53, M3:99, and M4:257, respectively; Fig. 1B). In contrast, the PTCs in exons 5, 6, and 7 (M5:308, M6:315, and M7:330, respectively) had little effect on mRNA abundance (Fig. 1B). Whereas the levels of HLA-A mRNA decreased between the transfecteds, they had comparable levels of endogenous HLA-A pre-mRNAs were detected in all the transfecteds. However, these species were more abundant in the transfecteds expressing mutants with PTCs in exons 2, 3, and 4 than in transfecteds expressing either normal A*2402 or mutants with PTCs in exons 5, 6, and 7 (Fig. 1B).

To investigate this difference further, fractions of nuclear and cytoplasmic RNA were prepared from the total RNA isolated from the transfecteds and separately analyzed on Northern blots (Fig. 1C). As expected, the higher m.w. bands corresponding to incompletely processed pre-mRNA were found only in the nuclear fractions. The abundance of these species was highest for the mutants with PTCs in exons 2, 3, and 4, and of intermediate level for the mutant with a PTC in exon 5. Normal A*2402 and the mutants with PTCs in exons 6 and 7 gave comparably low levels. The cytoplasmic fractions contained only mature mRNA, an inverse correlation being seen between the abundance of mature messenger in the cytoplasm and that of immature pre-mRNA species in the nucleus. These data are all consistent with the PTCs in exons 2, 3, and 4 triggering increased degradation of HLA-A transcripts by a process of NMD, a process well characterized for other mammalian and yeast genes (30–35).

From the positions of the PTCs, we predicted that the mutant proteins terminating in the extracellular domains (those encoded by mutants M2:53, M3:99, and M4:257) would associate with neither β2m nor cellular membranes. In contrast, the mutant proteins terminating in the transmembrane (M5:308) or cytoplasmic domains (M6:315 and M7:330) were predicted to associate with both β2m and cellular membranes. Although mutant M5:308 terminates in exon 5 that encodes the transmembrane domain, it does so at a site 3’ of the sequence encoding the hydrophobic transmembrane anchor. To test these predictions, we assessed the level of HLA-A protein at the surface of the transfected cells using the monomorphic HLA class I-specific Ab W6/32 (66) in flow cytometry (Fig. 1D). As expected, 721.221 cells transfected with M2:53, M3:99, and M4:257 gave no expression at the cell surface, whereas the cells transfected with M5:308, M6:315, and M7:330 gave levels of W6/32 reactivity similar to that obtained with the A*2402 control. For the latter mutants, the level of protein at the cell surface correlated well with the level of mRNA (Fig. 1, B and C).

IEF analysis of immunoprecipitates obtained with the W6/32 Ab demonstrated that 721.221 cells transfected with the M5:308 and M6:315 constructs expressed truncated proteins having isoelectric points identical to those predicted from their amino acid sequences (an isoelectric point of 5.81 for M5:308, and 5.99 for M6:315). Each of these mutant proteins was associated with β2m (Fig. 1E). As a control, similar immunoprecipitation analysis was performed with the polyclonal antiserum ABR2, which is specific for 10 aa (residues 330–339) in the cytoplasmic tail of HLA-A near the carboxyl terminus (67). Fig. 1E showed that wild-type A*2402 reacted with ABR2, whereas none of the A*2402 mutants reacted, as expected from the positions of the PTCs.

This set of mutants studied contained different nonsense codons as their PTCs, and all three types of nonsense codon were represented among the mutants. To assess whether the type of PTC affects mRNA level, we performed the following experiments. First, we made and compared a panel of mutants that all had TGA as their termination codons. This codon is most commonly used in human genes and terminates almost one-half of them (48%) (73); it is also the natural termination codon for HLA-A. In this panel of mutants all having TGA as the termination codon, the PTCs were attached to the same sites as those described in Fig. 1A. Northern blot comparison of mRNA levels in transfecteds expressing the two sets of mutants revealed no effects of different termination codons (data not shown). Second, all three types of termination codon were introduced at codon 99 in exon 3. All three mutants down-regulated the mRNA level to a similar extent, again showing no differential effect due to the type of PTC (data not shown).

Codons 275–295 in exon 5 of the HLA-A gene contain a transitional region for the induction of NMD

That termination at sites in exons 2, 3, and 4 gave dramatic reduction in mRNA level, while termination in exons 5, 6, and 7 had little effect, identifies the sequence from codon 257 (in exon 4) to codon 308 (in exon 5) as a region in which there is a transition in the effects of PTCs on mRNA level. To define more precisely this region of transition, additional mutants were made having PTCs at various positions in between codons 257 and 308 (Fig. 2A). These mutant genes were transfected into 721.221 cells, and HLA-A mRNA expression was analyzed by Northern blot (Fig. 2B).

Comparison of these mutants shows that PTCs at codons 295, 300, and 308 in the 3’ half of exon 5 have mRNA levels comparable with that of A*2402, although they differ from the control in having a characteristic pattern of immature pre-mRNA. In contrast, PTCs placed at the 3’ end of exon 4 and in the 5’ part of exon 5 reduced the mRNA level, with the greatest effects being seen for the two 5’-most PTCs, those causing termination at codons 274 and 275 (84% and 85% reduction, respectively). In mutant M4:275, the PTC is split between exons 4 and 5, but this arrangement...
did not interfere with its recognition, a phenomenon also reported from studies of the TPI and TCR-β chain genes (43, 49). These results further localize the region of transition within codons 275–295 in exon 5. They also show that there is a gradual decline in mRNA level as the PTC moved upstream in this region (~70% reduction with PTC 279 and 276, and up to ~85% reduction with PTC 275).

Experiments were also performed to determine the relative quantities of protein made in the transfectants shown in Fig. 2C. Analysis by flow cytometry showed that M5:308 was the only mutant that expressed W6/32-reactive HLA protein at the cell surface, which it did with an abundance comparable with the A*2402 control (Fig. 2C). Exon 5 encodes the transmembrane domain plus short, flanking, hydrophilic sequences. Of the mutations made in this panel, the PTC in mutant M5:308 is that in the 3′-most position, and its protein product includes all of the hydrophobic transmembrane anchor. The proteins encoded by the other mutants in this group lack part or all of this anchor, suggesting that their absence at the cell surface could have arisen because they do not associate with cell membranes. To test this hypothesis, we investigated whether the culture medium in which the transfectants had been grown contained soluble, secreted HLA-A molecules. This was done using a quantitative ELISA based upon the W6/32 Ab.

In comparison with untransfected 721.221 cells or cells transfected with the pHeBo vector alone, the supernatants from all transfected cells contained W6/32-reactive material (Fig. 2D). For A*2402 and mutant M5:308, the amounts were small, as both express membrane-bound proteins. By comparison, transfectants expressing mutants M5:295 and M5:300, which had mRNA levels comparable with the A*2402 transfectant, but no HLA-A protein at the cell surface, secreted high amounts of soluble HLA-A into the extracellular culture fluid. Mutants with a PTC at other upstream positions in exon 5 or at the 3′ end of exon 4 gave lower levels of soluble HLA-A, well correlated with their mRNA level. So, within this panel of mutants, the amount of protein made correlated with the level of mRNA, but its cellular location depended upon the placement of the PTC and its effect on membrane anchoring. The transition for membraneanchoring is relatively sharp, as removal of eight residues from the carboxyl-terminal end of the transmembrane region (the difference between M5:308 and M5:300) effectively converted the HLA-A molecules from a membrane-bound to a soluble form.

These experiments show that codons 275–295 in exon 5 contain a transitional region in which the effect of a PTC goes from causing 85% down-regulation of mRNA level to having negligible effect. A second critical region was also defined, one in which the PTCs do not lead to mRNA decay, but convert the protein from being membrane bound to being soluble and secreted. This region encompasses codons 295–300 and may extend into one or both flanking regions, although not beyond codons 279 and 308.

In the HLA-A gene, exon size and number both affect the location of the transitional region for NMD

The foregoing analysis demonstrates that PTCs on the 5′ side of exon 5 cause a major reduction in the steady state level of mRNA, whereas PTCs on the 3′ side of exon 5 do not. For PTCs within exon 5, there is a gradual transition between the two extremes. That these changes occur in the fourth-last exon contrasted with the results from DHFR, glutathione peroxidase 1, TCR-β, TPI, and β-globin genes, in which a boundary was found in the penultimate exon (37, 39, 40, 42, 44, 49, 54, 74).

To investigate this point further, we made and studied several artificial constructs of the HLA-A*2402 gene in which the length and content of the intron between exons 5 and 6 were changed. In the first set of constructs, an extra copy of exon 4 and its immediate flanking regions were inserted into the natural intron 5 (Fig. 3A). In these constructs, the fourth-last exon (the third exon upstream of the last exon/exon junction) was the extra exon 4 rather than the natural exon 5. Addition of this exon to the normal A*2402 gene (mutant E4) did not reduce the level of mRNA in transfected cells, although it was now longer due to the extra exon (Fig. 3B). Constructs containing PTCs in the extra exon 4 gave lower levels of mRNA in transfected cells, but with evidence of a gradient decreasing from 3′ to 5′ according to the position of the PTC (Fig. 3B). This is in contrast with PTCs in exon 4 of the normal HLA-A gene, in which they uniformly led to strong reduction of mRNA (~90% reduction in M4:257, M4:262, and M4:274). These data are consistent with a model in which the transition for mRNA decay induction of HLA-A is in the third exon upstream of the last exon/exon junction. Also supporting this model were the properties of a construct (M5:308,E4) in which the extra exon 4 was placed in the context of a PTC at codon 308 in exon 5 (Fig. 4A). The effect of the extra exon 4 was to convert this PTC from one that did not reduce the level of mRNA to one that did (88% reduction; Fig. 4B). The effect of the extra exon 4 was not simply due to an increase in size, because the addition of an intronic sequence of similar size did not convert PTC 308 (M5:308,13) into one that reduced mRNA level (Fig. 4).

As a further assessment of the model, the extra exon 4 in M5:308,E4 was replaced by a similarly sized fragment containing exon 6, intron 6, exon 7, and flanking regions to give mutant M5:308,E6/7 (Fig. 4A). If the region of transition in this construct localizes to the third exon upstream from the last exon/exon junction (the extra exon 7), then PTC 308 in exon 5 is predicted to strongly reduce the level of mRNA. The observed effect was partial reduction to a level one-third of that seen in the absence of extra exons (Fig. 4B). Thus, in this construct, the transitional region extends into the fifth exon upstream of the final exon/exon junction. This result indicates that the short exons 6 and 7, which are 33 and 48 nt in length, respectively, do not behave equivalently to the longer exon 4 (276 nt) in determining where PTCs will cause down-regulation of mRNA. It appears that the size of the exons is also a factor determining the site of transition. The small size of exons 6 and 7 in HLA-A could also explain why the transition region in the normal HLA-A*2402 gene is in the fourth-last exon, whereas in DHFR, glutathione peroxidase 1, TCR-β, TPI, and β-globin genes, it is the penultimate exon (37, 39, 40, 42, 44, 49, 54, 74).

Discussion

PTCs can arise in mRNA as a result of transcriptional error, somatic mutation, or the inheritance of germline mutation. The surveillance mechanism that causes mRNA-containing PTCs to be degraded has obvious benefits to cells and organisms; it prevents synthesis of mutant proteins that are inactive and would in some cases interfere with the function of the normal protein. The alleles and loci of the MHC class I family are noted for diversity and rapid evolution (75), processes that appear driven by the changing pressures imposed upon vertebrate populations by pathogenic microorganisms. For these genes, in which novelty appears frequently advantageous, it is necessary to understand the role that PTC-mediated mRNA degradation has upon their expression and evolution.

We have found that all PTCs placed in exons 2–4 encoding the extracellular domains of HLA-A caused ~90% reduction in the level of mature mRNA (Fig. 1). This degree of PTC-induced down-regulation is comparable with that found for the IgH chain gene (76), κ L chain gene (51, 53), and the TCR-β chain gene (50), but contrasts with reductions of 70–80% caused by PTCs in the
TPI, DHFR, and β-globin genes (37, 39, 40, 45). An impression gained from this admittedly small sample of genes is that diversifying genes of the adaptive immune system are under more stringent control by NMD than housekeeping genes. Our results also explain why many of the known natural HLA class I null alleles, having been defined by loss of cell surface antigenic phenotype, produce low or undetectable levels of mRNA: these alleles have PTCs in exon 1, 2, 3, or 4 (Fig. 5). It is therefore likely that any natural variant of an HLA class I gene having a PTC in exon 1, 2, 3, or 4 will be highly down-regulated at the mRNA level.

In our experiments, PTCs in exons 6 and 7 of HLA-A did not induce NMD, whereas PTCs in exon 5 had a variable effect that depended upon their positions. PTCs at the 5′ end of exon 5 gave maximal down-regulation of mRNA level; PTCs at the 3′ end had little effect. The region of transition was from codon 275 (85% down-regulation) to codon 295 (normal mRNA level). Analysis of other mammalian genes previously revealed this trend in which there is a boundary dividing the gene into two parts, a 5′ part in which PTCs induce NMD and a 3′ part in which they do not. From results obtained on the MUP, TPI, and β-globin genes, Nagy and...
Maquat (30) proposed a positional rule that defined the boundary as being 50–55 nt upstream of the 3' H11032-most exon/exon junction in the fully spliced mRNA (30). According to this rule, PTCs in the last exon and in the 3'-most 19 codons from the end of the penultimate exon should not induce NMD. An exception to this rule is the TCR-β-chain gene, for which down-regulation occurred for FIGURE 3. The mRNA-down-regulating effect of PTCs in exon 4 is ameliorated when the exon is artificially placed in the natural intron 5. A, Two types of mutant constructs of HLA-A*2402. First are mutants M4:257, M4:262, and M4:274, which have PTCs at positions 257, 262, and 274 of exon 4. Second are constructs E4:257, E4:262, and E4:274 in which the mutant exon 4 from mutants M4:257, M4:262, and M4:274 has been inserted as an extra exon within intron 5 of the normal A*2402 gene. As a control, E4 is a construct in which an additional exon 4 has been inserted into the intron. Exons containing a PTC are stippled. B, A Northern blot of total RNA isolated from 721.221 cells transfected with A*2402 mutants. Untransfected 721.221 cells and 721.221 cells transfected with vector alone were included as controls. The blot was probed sequentially for hyg', HLA-A, and β2m. HLA-A mRNA levels are expressed as a percentage of normal A*2402 or E4 mRNA, after normalization for the levels of hyg' mRNA.

FIGURE 4. Exon size and number influence PTC-mediated decay. A, Mutants M5:308,E4, M5:308,13, and M5:308,6/7 in which exon 4, intron 3, and exons 6 and 7 from the normal A*2402 gene have been inserted as an extra component within intron 5 of M5:308. As a positive control, E4 is a construct in which an additional exon 4 has been inserted into intron 5 of the normal A*2402 gene. As a negative control, M5:308,6:257 is a construct in which an additional exon 4 from mutant M4:257 has been inserted into intron 5 of M5:308. Exons containing a PTC are stippled. B, A Northern blot of total RNA isolated from 721.221 cells transfected with A*2402 mutants. Untransfected 721.221 cells and 721.221 cells transfected with vector alone were included as controls. The blot was sequentially probed for hyg', HLA-A, and β2m. HLA-A mRNA levels are expressed as a percentage of normal A*2402 or M5:308 mRNA, after normalization for the levels of hyg' mRNA.
from frameshift mutation by 1-bp deletion (b) being the allele designation in parentheses. PTCs in 11 alleles are generated (PTC: 264) (2). The positions of the PTCs are indicated followed, alleles have nucleotide substitutions that generate PTCs. m189) (19); j, B*5127N (PTC: 196) (11); q, B*5111N (PTC: 196) (13); r, A*2409N (PTC: 224) (11); s, A*0215N (PTC: 257) (5); and t, A*0243N (PTC: 264) (2). The positions of the PTCs are indicated following the allele designation in parentheses. PTCs in 11 alleles are generated from frameshift mutation by 1-bp deletion (a, b, l), 2-bp deletion (g), 1-bp insertion (n, o, p, q, i), 2-bp insertion (m), and 20-bp insertion (d); the PTCs generated are downstream of insertion/deletion event. The other nine alleles have nucleotide substitutions that generate PTCs.

transcripts containing PTCs in the penultimate exon at positions only 8–10 nt upstream from the 3’-most exon/exon junction (49). Neither do PTCs in the HLA-A gene conform to the positional rule, but in a way different from that seen in the TCR-β gene. According to the positional rule, the transitionary boundary of HLA-A should reside at codons 323–324 in exon 6. In our experiments, PTCs in the last three exons (6, 7, and 8) of HLA-A did not induce NMD, the boundary being situated >58 nt upstream of the third-last (exon 5/ exon 6) rather than the ultimate, exon/exon junction (Fig. 6).

That the boundary in the HLA-A gene is located ~58 nt upstream of an exon/exon boundary suggests that the scanning machinery recognizes PTCs similarly in the HLA-A, MUP, TPI, and β-globin genes (30). What distinguishes HLA-A is that the junctions between exons 6 and 7 and between exons 7 and 8 do not appear to count. Exons 6 and 7 in HLA-A are unusually small exons, comprising just 33 and 48 nt, respectively. That these exons are shorter than 50–55 nt might explain why PTCs are recognized differently than according to the prevailing paradigm. Emphasizing the distinct behavior of the short exons was our observation that the presence of four short downstream exons did not move the transition region completely out of exon 5 (Fig. 4). In contrast, insertion of an additional, longer exon into intron 5 moved the transition region out of exon 5 and downstream into the inserted exon (Fig. 3). One possibility is that the boundaries involving such short exons are not marked in the nucleus in the same way as those involving longer exons, and that this makes them invisible to the scanning machinery. Additionally, the close juxtaposition of PTCs in short exons and the marks that define the exon junctions may prevent effective recognition by the scanning machinery. The latter possibility is compatible with the hypothesis of Zhang et al. (42) that 50–55 nt represents the minimum separation needed for the scanning machinery to recognize both a PTC and a downstream mark.

In classical MHC class I genes, the cytoplasmic tails are always encoded by a number of short exons. Thus, the results we obtained for HLA-A are likely to have generality for other classical class I genes. A corollary of the positional rule is that when the natural termination codons are not encoded in the last exon, it lies no further than 50–55 nt upstream of the 3’ end of the penultimate exon. Of the genes surveyed by Nagy and Maquat (30), only two are reported to fail this rule, one of these being the relatively non-polymorphic, human MHC class I gene HLA-G (64); however, this might not be entirely true for HLA-G. Although the genomic organization of HLA-G is homologous to that of HLA-A, the homologous HLA-G exon 7 has not been found in any reported cDNA sequenced (77). An alternative stop codon in exon 6 is also used for the natural termination of HLA-G. Thus, the exon 6/ exon 8 junction is effectively the 3’-most junction, which placed the natural stop codon in exon 6 at a position only 31 nt upstream of the ultimate exon/exon junction, and thus adhering to the positional rule of Nagy and Maquat (30). As such, the observations with our HLA-A mutants are even more remarkable.

From our analysis, the functional possibilities of variant HLA class I genes containing PTCs appear to depend dramatically upon where the PTC is located. Variant alleles having PTCs in exons 1–4, the highly polymorphic part of the gene, would be largely shut down by NMD. Moreover, almost all such variants would not encode class I H chains that associate with β2m and bind peptides. Such mutations could be the substrate for positive selection under circumstances in which a class I allele or locus is having a deleterious effect on host survival and reproduction. The HLA-H and J loci appear to be examples of class I genes that were once functional for which alleles containing PTCs have been fixed either through selection or genetic drift (78, 79). However, in most circumstances, it is expected that alleles with PTCs in exons 1–4 will lack advantage and may diminish immune responsiveness: heterozygotes will become effective homozygotes for the other functional allele, and homozygotes will lack the locus altogether. This need not be obviously detrimental, as evidenced by apparently healthy homozygotes for HLA-A*0215N (5) and HLA-B*1526N (12).
Variant alleles with PTCs in exons 6 and 7 encoding the cytoplasmic domain are expressed at the cell surface at normal levels and differ solely in the length and sequence of their cytoplasmic tails. Natural variants of this type have not been described, but that does not mean they do not exist: neither the serological nor DNA typing methods used to screen populations for HLA class I probe for polymorphism in exons 5, 6, 7, and 8. It is difficult to assess the possible functional effects of cytoplasmic tail mutants upon which natural selection might operate, because so little is known of the purpose of this part of the MHC class I molecule (80). Recently, Cohen et al. (81) demonstrated that the cytoplasmic tail is necessary for the Nef-dependent down-regulation of HLA-A and B during HIV infection, illustrating how it can be used to a pathogen’s advantage. This clearly raises the possibility that there are infections in which novel changes in the cytoplasmic tail of an MHC class I molecule confer an advantage to the host. The normal, early termination in HLA-G also supports this conjecture.

Variant alleles with PTCs in exon 5 have perhaps the potential for the widest ranging effect. Mutation throughout this exon produces variant class I H chains that associate with peptides. With the exception of the few 3’/H11032

References