SECRETION OF HLA-A AND -B ANTIGENS VIA AN ALTERNATIVE RNA SPLICING PATHWAY

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Class I molecules of the MHC are polymorphic cell surface glycoproteins that appear to mediate interactions between CTL and their target cells (1). They are heterodimers consisting of a 43,000-D, MHC-encoded heavy chain, noncovalently associated with a 12,000-D light chain, β_2-microglobulin (2). This complex is anchored in the membrane via a stretch of hydrophobic amino acids near the carboxyl terminus of the heavy chain, and as such has properties typical of many integral membrane glycoproteins.

Class I genes are comprised of eight exons, which correlate well with structural and functional domains of the molecule (3). Exon 1 encodes the signal peptide, exons 2–4 encode three extracellular domains, exon 5 encodes the transmembrane region, and exons 6, 7, and (in most cases) 8 encode a small, intracellular tail.

Previous studies of a mutagenized B lymphoblastoid cell line have identified a clone, 8.14.1, which secretes a water soluble form of the HLA-A2 antigen (4, 5). Characterization of the secreted polypeptide, and ultimately, the analysis of cDNA clones from both the parent and mutant cell lines, indicate that secretion results from an unusual pattern of RNA splicing that excludes exon 5 from mature, HLA-A2–encoding transcripts (6). Instead, exon 4 is joined directly to exon 6, with the correct reading frame maintained. A transcript spliced in this fashion would be expected to encode a polypeptide that lacks 39 amino acids, including the hydrophobic amino acids that would normally anchor the polypeptide in the plasma membrane. However, the polypeptide would retain a normal carboxyl terminus.

Although class I molecules are best understood in terms of both structure and function as cell surface integral membrane glycoproteins, there have been reports of soluble forms of these molecules. For example, HLA-A and -B antigenic determinants have been detected in high speed supernatants of human serum (reviewed in reference 7). Emerson et al. showed that H-2K^k is shed in association with membrane lipids from murine spleen cells (8). Most recently, a gene in the Qa region of the murine MHC has been shown to encode a truncated class I molecule that is synthesized in the liver and is detectable in the serum (9–12).

The example of mutant 8.14.1 has suggested a potential mechanism for generating stable, water soluble, secreted forms of HLA-A and -B antigens in vivo. Using mutant 8.14.1 and its parent cell line T5-1 as a model system, the

present study was conducted to assess whether a similar splicing mechanism might be operative in generating secreted HLA-A and -B locus products in nonmutagenized human cells. T and B cell lines, a hepatoma cell line, and normal PBL are examined.

Materials and Methods

Cells and Cell Culture. B lymphoblastoid cell lines used were: T5-1 (HLA-A1, -A2, -B8, -B27) and its immunoselected mutant 8.14.1, WT51 (HLA-A9, -B14), MST (HLA-A3, -B7), JY (HLA-A2, -B7), SB (HLA-A1, -A2, -B12, -Bw17), AS (HLA-A2, -A24, -B27), and 23.1 (HLA-A2, -B27). T leukemic cell lines used were: HPB-ALL (HLA-A24, -B15, -Bw51), HPB-MLT (HLA-A24, -Aw33, -Bw15), MOLT-3 (HLA-A1, -A10), 8402 (HLA-A1, -A29), CEM (HLA-A1, -Aw30, -B8, Bw40), and HSB (HLA-A1, -A2, -B12, -Bw17). The hepatoma cell line used was HEP-G2 (HLA-A2, -A9, -B5, -Bw17). Cells were maintained in either RPMI 1640 or DME (Gibco Laboratories, Grand Island, NY), supplemented with either 10 or 15% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. PBL were obtained from donors M.B. (HLA-A1, -A24, -B18, -Bw44), J.C. HLA-A2, -A23, -B27, -Bw44), and M.L. (HLA-A2, -A11, -B7, -Bw35) by centrifugation through Lymphocyte Separation Medium (Litton Bionetics, Kensington, MD). 3-d PHA- (Difco Laboratories Inc., Detroit, MI) stimulated cultures were harvested for metabolic labeling.

Metabolic Labeling, Preparation of Cell Lysates, and Culture Supernatants. Cells were preincubated for 45 min in methione-free RPMI 1640 or DME supplemented with 10% FCS at a density of 2×10^6/ml, and were then labeled in this same medium supplemented with 250 μCi/ml [^{35}S]methionine (1,000 Ci/mmol, New England Nuclear, Boston, MA) for a period of 4–14 h. After labeling, cells were harvested by centrifugation for 5 min at 1,000 g. Cells were washed once in PBS, and were lysed at 4×10^6/ml in the presence of 2% NP-40 (Particle Data, Inc., Elmhurst, IL), 0.5 mM DTT, 0.1 mM PMSF, 10 mM Tris-HCl (pH 7.5). After 0.5–1 h on ice, lysates were cleared by centrifugation at 13,000 g for 15 min in an Eppendorf microfuge. Labeling supernatants were cleared in similar fashion, and these low speed supernatants were usually centrifuged for an additional 1 h at 100,000 g using a 50Ti rotor (Beckman Instruments, Inc., Palo Alto, CA) in either a Beckman L5-65 or Sorvall OTD-65 ultracentrifuge (Du Pont de Nemours, E.I. & Co. Inc./Sorvall Instruments Div., Newton, CT). Lysates and culture supernatants were routinely stored at −70°C.

Antibodies and Immunoprecipitation. mAbs used were: W6/32 (HLA-A–, -B–, -C–specific; reference 13); 4E (HLA-B–, -C–specific; reference 14); PA2.1 (HLA-A2–specific; reference 15); and 187.1 (mouse kappa chain–specific, a gift from Dr. Brett Spear, University of Pennsylvania, Philadelphia, PA). Aliquots of lysate and supernatant were precleared by incubation with normal rabbit serum plus an excess of *Staphylococcus aureus* immunoadsorbent (Pansorbin; Calbiochem-Behring, La Jolla, CA) for 2 h on ice. Protein A Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ) was then used as the immunoadsorbant for both the control and specific antibody immunoprecipitations presented. Conditions for washing immunoprecipitates were as described (16), except that only three high salt and one low salt washes were performed.

Gel Electrophoresis. Analyses of polypeptides by discontinuous SDS-PAGE and by two-dimensional gel electrophoresis were as described previously (5, 17, 18). Radioactive species were detected by fluorography (19), and quantitation of fluorographic or autoradiographic images was performed using a Quick Scan R + D densitometer (Helena Laboratories, Beaumont, TX).

Detergent Binding and Proteolytic Digestion Analyses. Precondensed Triton X-114 (TX-114;[1] Fluka, Hauppauge, NY) was a generous gift from Dr. Carolyn Doyle, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. TX-114 was used in place of NP-40 for cell lysis, and was added to culture supernatant preparations at 1% final concentration. Condensation and phase separation were performed as described by Bordier (20). Prote-

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[1] *Abbreviation used in this paper:* TX-114, Triton X-114.

olysis with both papain and *Pseudomonas fragi* protease was performed essentially as described previously (5). However, papain digestion of TX-114 solubilized cell extracts was performed at 14°C for 3 h to avoid condensation during the incubation.

Ultracentrifugal Analyses. Ultracentrifugal flotation was based upon the conditions of Allison et al. (21). Briefly, solid KBr was added to 1-ml aliquots of a culture supernatant preparation to bring their densities to either 1.06 or 1.21 g/ml. 0.65-ml aliquots were then centrifuged for 44 h at 43,000 rpm in 5 × 41-mm ultraclear centrifuge tubes, using an AH650 rotor with appropriate adaptors in a model OTD 65B ultracentrifuge (Du Pont de Nemours, E.I. & Co. Inc./Sorvall Instruments Div.). Three 0.2-ml fractions were harvested by pipetting from the top of the tube. These were diluted up to 1 ml with the addition of 10 mM Tris-HCl (pH 8.0), after which immunoprecipitation was performed as usual. Sucrose density gradient centrifugation was performed using a 12-ml 5–20% gradient of sucrose in 0.15 M NaCl, 5 mM EDTA, 0.2% azide, 50 mM Tris-HCl (pH 7.5). 0.6 ml of culture supernatant preparation was mixed with ^{125}I-labeled BSA (a gift from Dr. John Fraser, Dana Farber Cancer Institute) to be used as a marker, and was loaded onto the gradient. Centrifugation was for 20 h at 26,000 rpm using a model SW50 rotor in a model L5-65 centrifuge (Beckman Instruments Inc.). 0.7-ml fractions were collected using a peristaltic pump, and were used directly for immunoprecipitation analysis. In addition, small aliquots of each fraction were used to determine the distribution of ^{125}I radioactivity across the gradient.

Purification and Analysis of RNA. Enrichment for poly(A)-containing RNA, agarose gel electrophoresis, and blot hybridization analysis were carried out as described previously (6). The A4/6 20mer and an HLA-B7 cDNA insert (22) were used as probes under conditions described previously (6).

Identification and Sequence Analysis of cDNA Clones. A lambda gt10 cDNA library constructed from HPB-MLT poly(A) + RNA was kindly provided by Dr. Deno Dialynas, Harvard University, Cambridge, MA (Dialynas, D., C. Murre, T. Quertermous, J. M. Boss, J. G. Seidman, and J. L. Strominger, manuscript submitted for publication). Plaque lifts were screened in duplicate, with the HLA-B7 cDNA and oligonucleotide A4/6 under conditions decribed previously (6). Phage minipreps were essentially as described (23). Inserts were purified by electrophoresis through low melting-point agarose (Bethesda Research Laboratories, Gaithersburg, MD), subcloned into the Eco RI site of pBR322, and plasmid minipreps prepared (24). Pvu II digested DNA was cloned into the Sma I site of M13 mp18, and was sequenced by the dideoxy chain termination method (25), using a 6% polyacrylamide gel with a buffer gradient (26).

Results

HLA-A and -B Antigens Are Detectable in Culture Supernatants of a T Leukemic Cell Line. The T leukemic cell line HPB-ALL (HLA-A24, -B15, -Bw51; reference 27) has been characterized in detail. Cells were metabolically labeled for 14 h with [^{35}S]methionine, after which detergent lysates were prepared, and culture supernatants were harvested, as described in Materials and Methods. Immunoprecipitation was performed using the HLA-A–, -B–, -C–specific mAb W6/32, and immunoprecipitated polypeptides were resolved by SDS-PAGE and visualized by fluorography (Fig. 1*A*). Whereas polypeptides of 43,000 and 12,000 D are detected in the cell lysate, polypeptides of 39,000 and 12,000 D are detected in the culture supernatant. A similar experiment performed after labeling in the presence of tunicamycin rules out the possibility that the unusual size of the supernatant localized form results from a glycosylation difference (data not shown). Taking into account the difference in cell lysate and culture supernatant cell equivalents used in the experiment presented in Fig. 1*A*, densitometric quantitation suggests that the 39,000-D species is present at a level ~2.5% that of the 43,000-D species. Over the course of many experiments this number has
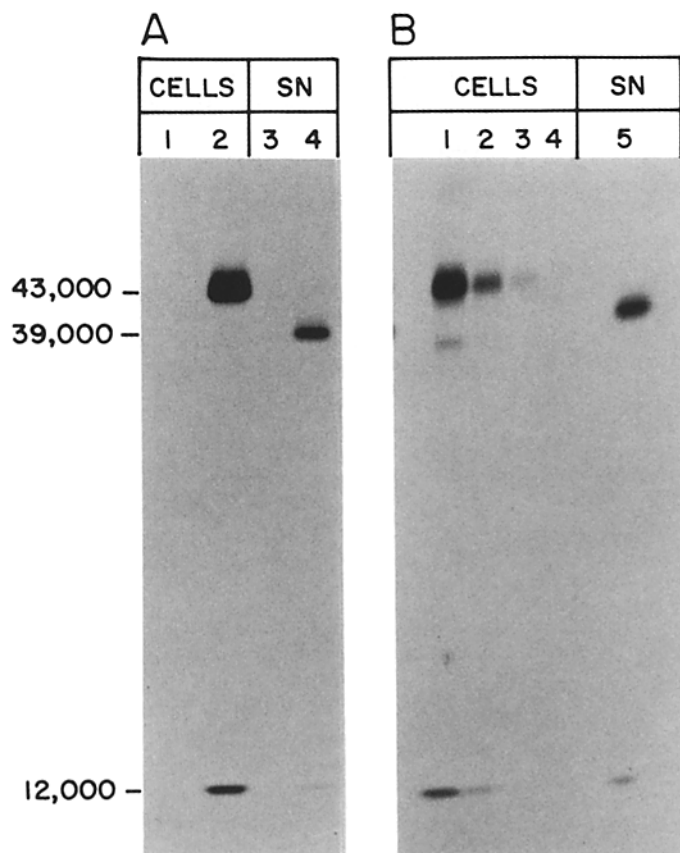
FIGURE 1.   Immunoprecipitation analysis of HPB-ALL cell lysates and culture supernatants. (A) Cell lysate and culture supernatant were prepared from cells labeled for 14 h in the presence of [³⁵S]methionine. Cell lysate immunoprecipitations were from 0.2 times the cell equivalents used for the supernatant immunoprecipitations. The antibodies used were: lanes *1* and *3*, normal rabbit serum; lanes *2* and *4*, W6/32. (B) Cell lysate and culture supernatant were prepared from cells labeled for 4.5 h in the presence of [³⁵S]methionine. Immunoprecipitation was with W6/32. Lanes *1–4* are serial threefold dilutions of immunoprecipitated material, representing 0.1, 0.03, 0.01, and 0.003 times the cell equivalents used for the supernatant immunoprecipitation (lane *5*). Densitometric analysis indicates a near linear response in the intensity of the 43,000-D species over the range covered by the dilutions in lanes *1–3*. Analysis was by SDS-PAGE followed by fluorography, and only the relevant portion of the fluorograph is presented.

varied from 1 to >5%, with labeling times varying between 4 and 14 h. In the experiment depicted in Fig. 1 *B*, a 4.5-h labeling period reveals that polypeptides of ~39,000 D can be detected in both the culture supernatant and the cell lysate. This cellular form is typically found in shorter labeling periods, and presumably represents an intracellular precursor to the supernatant localized form. Most often, the total amount of extracellular and intracellular 39,000-D forms is ~4% that of the 43,000-D form. However in this particular experiment, the extracellular form represented ~5%, and the intracellular form ~11%. The reason for this variability is not understood.

Fig. 2 presents two-dimensional gel electrophoretic analysis of immunoprecip-

CELLS   SUPERNATANT

+   IEF   −

W6/32   SDS-PAGE

A   C

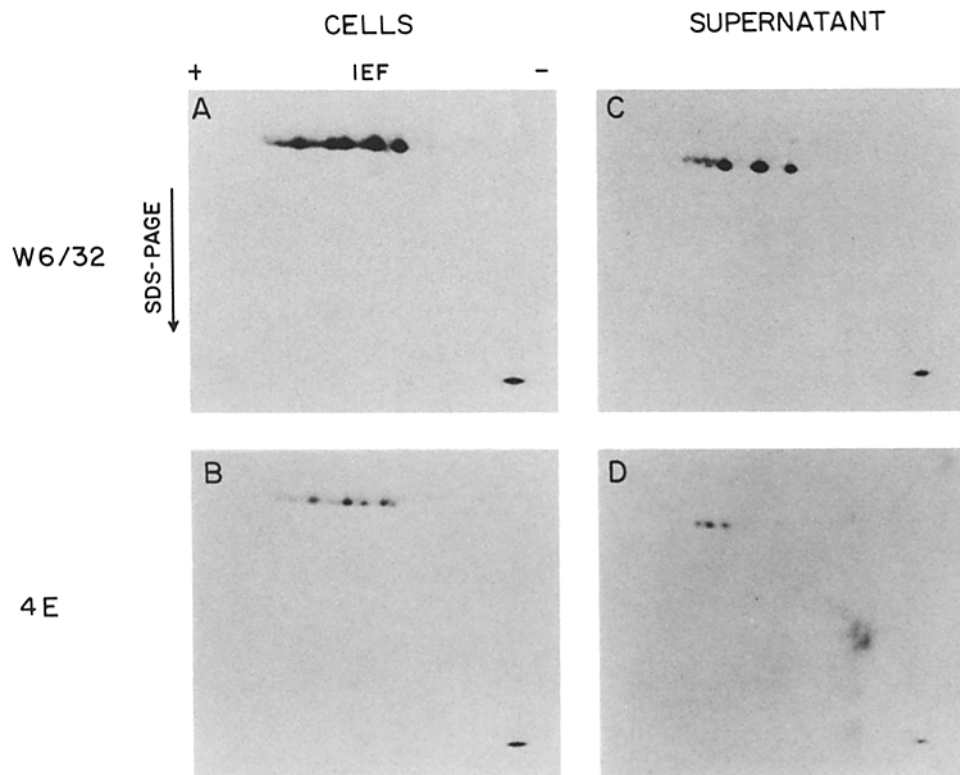B   D

4E

FIGURE 2. Two-dimensional gel electrophoresis of immunoprecipitates from HPB-ALL cell lysate and culture supernatant. Only the relevant portions of the fluorographs are presented. Cell lysate and culture supernatant immunoprecipitations are not quantitatively related. The antibodies used were: W6/32 (HLA-A-, -B-, -C-specific), A and C; and 4E (HLA-B-, -C-specific), B and D. Those spots immunoprecipitated by W6/32 but not by 4E are presumed to represent HLA-A24.

itated polypeptides. The 39,000-D polypeptides immunoprecipitated by W6/32 from the HPB-ALL culture supernatant are clearly resolved into two series of spots (Fig. 2C). Immunoprecipitation using an antibody specific for HLA-B and -C locus products (4E) indicates that the less abundant spots represent HLA-B and/or -C locus products (Fig. 2D), whereas the more abundant spots represent A locus products. Thus, both an HLA-A and an HLA-B (or -C) locus product are present in the HPB-ALL culture supernatant. The relative abundance of the two products has not been found to vary.

The Supernatant Class I Molecules Behave As Water Soluble Heterodimers. Culture supernatant–localized class I molecules could potentially arise by a number of distinct mechanisms, including proteolysis from the cell surface, shedding in association with membrane lipid, and bona fide secretion of water soluble forms. The structure and characteristics of the supernatant molecules were studied to help distinguish among these possibilities.

The solubility properties of the supernatant-localized molecules were investigated by assessing their interaction with the detergent TX-114. At 4°C, a solution of TX-114 is homogeneous, whereas at 37°C this solution separates into a
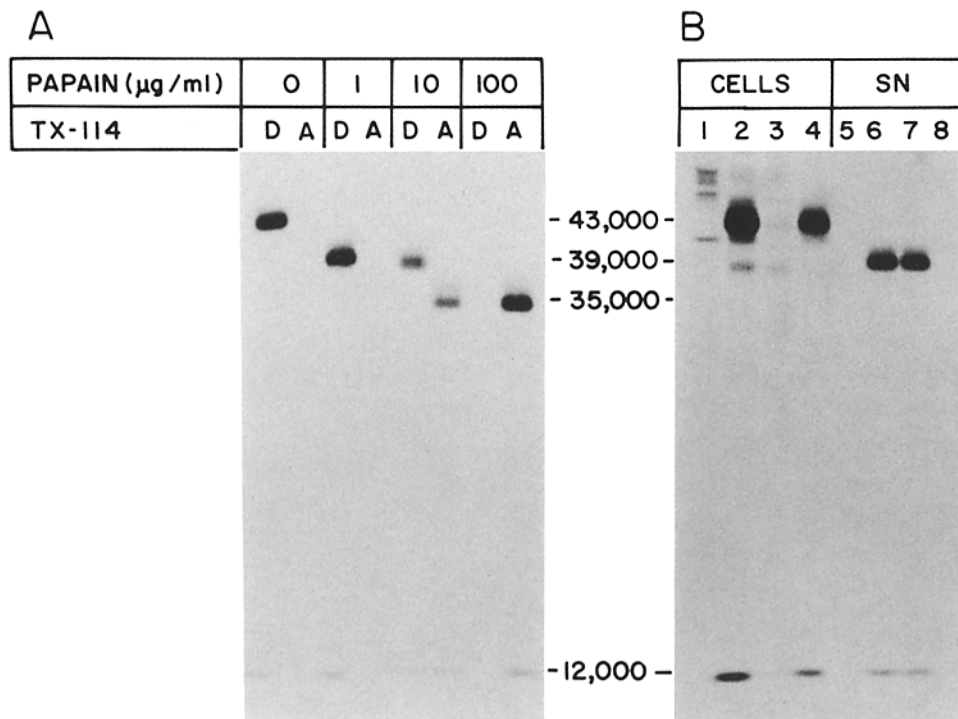
A

PAPAIN (μg/ml)	0		I		10		100	
TX-114	D	A	D	A	D	A	D	A

B

	CELLS				SN			
	I	2	3	4	5	6	7	8

- 43,000 -

- 39,000 -

- 35,000 -

- 12,000 -

FIGURE 3. Detergent binding properties of immunoprecipitated polypeptides. HPB-ALL cells were labeled for 4 h in the presence of [^{35}S]methionine, after which the culture supernatant was harvested and cell lysate was prepared using either TX-114 or NP-40 (A) Aliquots of TX-114 cell lysate were treated with increasing amounts of papain, after which they were separated into detergent (D) or aqueous (A) phases. Immunoprecipitation was then performed using W6/32, and immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. (B) Aliquots of NP40 cell lysate were immunoprecipitated using either normal rabbit serum (lane 1) or W6/32 (lane 2). An equivalent aliquot of TX-114 cell lysate was separated into aqueous (lane 3) and detergent (lane 4) phases, which were then immunoprecipitated using W6/32. Aliquots of culture supernatant were immunoprecipitated with either normal rabbit serum (lane 1) or W6/32 (lane 2). TX-114 was added to an equivalent aliquot of culture supernatant, after which it was separated into aqueous (lane 7) and detergent (lane 8) phases, which were then immunoprecipitated using W6/32. Cell lysate immunoprecipitations were from 0.1 times the cell equivalents used for culture supernatant immunoprecipitations.

detergent phase and an aqueous phase. Molecules that bind detergent, such as integral membrane proteins, will usually partition into the detergent phase, whereas those that are water soluble will partition into the aqueous phase (20). To test whether this expectation holds true for class I molecules, a control experiment was performed. The protease papain is known to generate two discrete proteolytic fragments of the heavy chain, a 39,000-D fragment that lacks the carboxy terminal hydrophilic tail but retains the hydrophobic region, and a 35,000-D fragment that lacks both of these segments of the polypeptide (28). In Fig. 3A, increasing amounts of papain were used to convert the 43,000-D cellular form of the HPB-ALL class I polypeptides successively to 39,000- and then 35,000-D forms. It was found that whereas the 43,000- and 39,000-D species partitioned into the TX-114 detergent phase, the 35,000-D species partitioned

into the aqueous phase. Thus, as expected, those fragments that retained the hydrophobic region bound detergent, whereas that that had lost the hydrophobic region failed to bind detergent.

Fig. 3B shows that the 39,000-D, supernatant-localized polypeptides fail to bind detergent, since they partition into the TX-114 aqueous phase (lanes 5–8). This is also the case for the 39,000-D, cellular polypeptides (lanes 1–4), supporting the argument that they are related to, and hence likely represent intracellular precursors of, the supernatant forms. These detergent-binding properties contrast sharply with those of the 39,000-D proteolytic fragment of the heavy chain (Fig. 3A), indicating that the 39,000-D supernatant-localized polypeptides are not proteolytic fragments of the membrane forms. Separate experiments based upon the sedimentation of culture supernatant material through sucrose density gradients indicate that the supernatant-localized molecules behave as simple heavy chain–light chain heterodimers, and thus are not present in any larger aggregates (data not shown). Together, these experiments provide evidence that the supernatant-localized molecules are water soluble heterodimers, that they do not arise from the cellular forms by a simple truncation at the carboxy terminus, and that they probably do not contain a functional hydrophobic region. Thus they appear to be truly secreted forms of class I molecules, as distinguished from proteolyzed or shed forms. In all of these properties they appear closely related to the HLA-A2 molecules secreted by mutant 8.14.1.

Structural Analysis Indicates an Internal Deletion Near the Carboxyl Terminus. More direct information regarding the physical structure of the secreted molecules was obtained by proteolytic cleavage analysis of the carboxyl terminus. As noted above, proteolysis using the enzyme papain is known to degrade the 43,000-D heavy chain to a 39,000-D fragment that has lost the carboxy terminal hydrophilic tail, and a 35,000-D fragment that has in addition lost the hydrophobic region. As shown in Fig. 4A, these cleavages occur normally for the HPB-ALL cellular class I antigens. However, they do not occur normally for the HPB-ALL–secreted class I antigens (Fig. 4B). In this case, an initial cleavage to ~38,000 D is observed, which is then followed by a cleavage to a 35,000-D limit product. The initial cleavage that removes 1,000 D has been observed previously for the membrane form, and is therefore not regarded as a significant departure from the norm (5). However, the fact that the limit papain products of the cellular and secreted forms are of similar size implies that the structural difference between the cellular and secreted forms resides somewhere within their carboxy termini. It is particularly striking that the cleavage pattern for the HPB-ALL–secreted molecule is identical to that observed for the HLA-A2 molecule secreted by mutant 8.14.1 (Fig. 4C). Further analysis using *P. fragi* protease is presented in Fig. 4, D–F. This protease is known to cleave the class I antigen heavy chain within the carboxy terminal hydrophilic region, and hence should serve as a marker for the presence of this portion of the polypeptide (29). The results clearly map *P. fragi* protease cleavage sites to identical distances from the carboxy termini of the cellular and secreted molecules. Again, the cleavage pattern for the HPB-ALL–secreted molecule is identical to that for the HLA-A2 molecule secreted by mutant 8.14.1. The mapping of these proteolytic cleavage sites has previously been interpreted as predictive of an internal deletion removing the
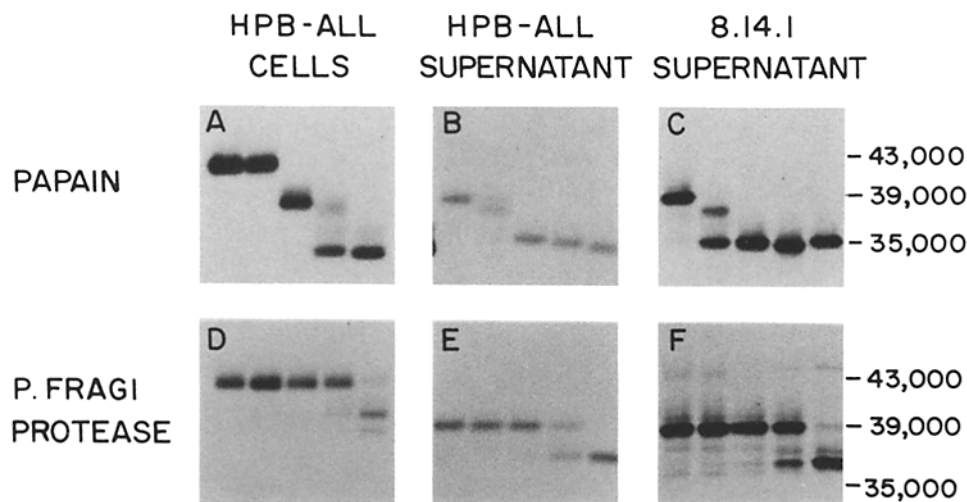
HPB-ALL   HPB-ALL   8.14.1
CELLS   SUPERNATANT   SUPERNATANT

FIGURE 4.   Proteolytic digestion analysis of cell lysate and culture supernatant class I poly-peptides. HPB-ALL and 8.14.1 cells were labeled in the presence of [^{35}S]methionine, and cell lysates and culture supernatants were prepared. Aliquots of HPB-ALL cell lysate (A and D), HPB-ALL culture supernatant (B and E), and 8.14.1 culture supernatant (C and F), were treated with varying amounts of either papain or $P.$ $fragi$ protease, precisely as described previously (5). Immunoprecipitation was performed using W6/32, and immunoprecipitated polypeptides were analyzed by SDS-PAGE followed by fluorography. Within each panel, the leftmost lane represents a control incubation in the absence of protease. From left to right, papain concentrations were 0.1, 1.0, 10.0, and 100.0 μg/ml, and $P.$ $fragi$ protease concentrations were 11.2, 56.0, 280.0, and 1400.0 μg/ml.

transmembrane hydrophobic region, rather than a simple truncation removing the carboxy terminal hydrophilic peptide (5). Hence these structural data are consistent with the functional data obtained based upon TX-114 binding.

Detection of Alternatively Spliced Transcripts Encoding HLA-A24.   The internal deletion suggested by the above data could be explained by an alternative splicing model, in which the secreted molecules would derive from transcripts from which exon 5 had been removed, whereas the membrane forms would derive from transcripts that retain this exon. To provide direct evidence for such splicing patterns, two types of experiments were performed. Both experiments rely on the use of a synthetic oligodeoxyribonucleotide (A4/6), which consists of the last ten nucleotides of exon 4 juxtaposed with the first ten nucleotides of exon 6, on the noncoding strand of the HLA-A2 gene. Previous experiments using mutant 8.14.1 and its wild-type parent cell line T5-1 have shown that A4/6 can be used to probe Northern blots to selectively detect transcripts that lack exon 5 (6). Further, this oligonucleotide could also be used to detect cDNA clones derived from such transcripts. In Fig. 5, RNA samples isolated from T5-1, 8.14.1, and HPB-ALL are resolved by agarose gel electrophoresis, transferred to nitrocellulose, and probed with either a ^{32}P-labeled cDNA probe which should hybridize to all class I antigen encoding transcripts, or ^{32}P-labeled oligonucleotide A4/6. The cDNA probe detects transcripts present in all three cell lines (Fig. 5A). The oligonucleotide, however, detects transcripts present in 8.14.1 and HPB-ALL, but absent from T5-1 (Fig. 5B). The detection of these transcripts provides
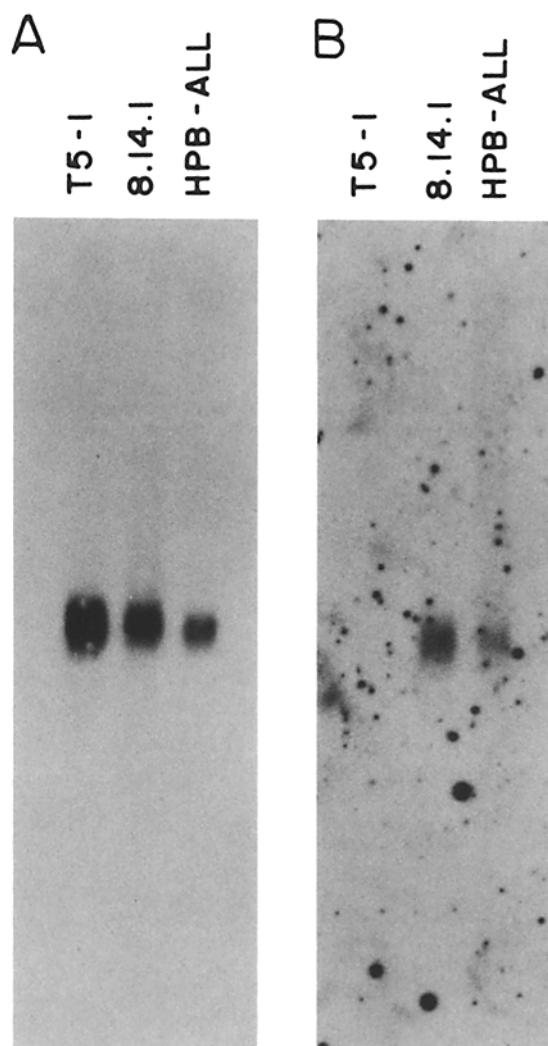
FIGURE 5. Northern blot analysis of T5-1, 8.14.1, and HPB-ALL class I transcripts. Poly(A) + RNA isolated from T5-1, 8.14.1, and HPB-ALL was fractionated on a 1.5% agarose gel, transferred to nitrocellulose, and hybridized with either the ^{32}P-labeled HLA-B7 cDNA probe (A) or ^{32}P-labeled oligonucleotide A4/6 (B). The same nitrocellulose filter was used with both probes.

strong evidence that the proposed splicing pattern indeed occurs in the HPB-ALL cell line.

It remained to be proven that transcripts encoding a single allelic product could be spliced in both fashions, and that the proposed structure of the secreted polypeptides was fully consistent with the structure of the unusually spliced transcripts. To do so, both oligonucleotide A4/6 and the cDNA probe were used in a double screen of a cDNA library constructed in the lambda gt10 vector, using RNA isolated from the cell line HPB-MLT (Dialynas, D., C. Murre, T. Quertermous, J. M. Boss, J. G. Seidman, and J. L. Strominger, manuscript

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                    250                                                   260
clone 5    CT GTG GTG GTA CCT TCT GGA GAG GAG CAG AGA TAC ACC TGC CAT GTG CAG CAT GAG GGT
clone 1    CT GTG GTG GTA CCT TCT GGA GAG GAG CAG AGA TAC ACC TGC CAT GTG CAG CAT GAG GGT

                    270                      E4E5                280
clone 5    CTG CCC AAG CCC CTC ACC CTG AGA TGG GAG CCA TCT TCC CAG CCC ACC GTC CCC ATC GTG
clone 1    CTG CCC AAG CCC CTC ACC CTG AGA TGG G-- --- --- --- --- --- --- --- --- --- ---

                    290                                                   300
clone 5    GGC ATC ATT GCT GGC CTG GTT CTC CTT GGA GCT GTG ATC ACT GGA GCT GTG GTC GCT GCT
clone 1    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---

                    310                      E5E6                320                   E6E7
clone 5    GTG ATG TGG AGG AGG AAC AGC TCA GAT AGA AAA GGA GGG AGC TAC TCT CAG GCT GCA AGC
clone 1    --- --- --- --- --- --- --- --- -AT AGA AAA GGA GGG AGC TAC TCT CAG GCT GCA AGC

                    330                                      340E7E8
clone 5    AGT GAC AGT GCC CAG GGC TCT GAT GTG TCT CTC ACA GCT TGT AAA GTG TGAGACAG
clone 1    AGT GAC AGT GCC CAG GGC TCT GAT GTG TCT CTC ACA GCT TGT AAA GTG TGAGACAG
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FIGURE 6. Partial nucleotide sequences of clones 1 and 5. Sequences are divided into triplet codons which are numbered from the amino terminus of the mature protein. Exon boundaries are marked, i.e., E4E5. (−) indicates nucleotides encoded within exon 5 that are absent from clone 1.

submitted for publication). Although HPB-ALL and HPB-MLT are documented to be of independent origin (27), the stocks presently available appear to be identical. As evidence for this, both cell lines react with the T40/25 clonotypic anti–T cell receptor antibody (30), both appear to have undergone identical T cell receptor beta chain gene rearrangements (Leiden, J. M., D. P. Dialynas, A. D. Duby, C. Murre, J. Seidman and J. L. Strominger, manuscript submitted for publication), and both express identical arrays of cellular and secreted class I polypeptides, as analyzed by two-dimensional gel electrophoresis (data not shown).

Duplicate filters carrying ~300,000 plaques were screened with each probe. Of the ~60 plaques found to hybridize with the cDNA probe, one also hybridized with oligonucleotide A4/6. It is likely that this represents, if anything, an underestimate of the relative abundance of unusually spliced transcripts, because the cDNA probe would be expected to hybridize with clones derived from unusually spliced transcripts that might not be long enough to include the sequence complementary to the oligonucleotide. Clone 1, which was cDNA+ and A4/6+, and five randomly selected cDNA+ and A4/6− clones were analyzed in detail. Since HPB-ALL secretes primarily its A locus product, HLA-A24, it was deemed likely that clone 1 would represent an HLA-A24–encoding cDNA, as would at least some of the randomly selected clones. It has so far been possible to distinguish HLA-A− from HLA-B–encoding cDNAs by the presence of an internal 165-bp Pst I fragment in the former (6, 31). Clone 1, along with clones 5 and 7, were found to contain such a fragment. Based upon the sequence of the HLA-A24 gene, Pvu II sites would be expected in the middle of exon 4, and at the beginning of exon 8 (32). Thus Pvu II digestion should yield fragments of different size, dependent upon the splicing pattern within this region. Whereas fragments of 290 bp were generated from clones 5 and 7, a fragment of 165 bp was generated from clone 1. The clone 1 and clone 5 Pvu II fragments were then subcloned and their sequences determined.

Fig. 6 presents the nucleotide sequences of the clone 1 and clone 5 Pvu II fragments. Both clones begin within codon 246 and end a few nucleotides into

the 3' untranslated region. The sequence of clone 5 is identical to that reported for the same region of the HLA-A24 gene (32). The sequence of clone 1 differs only in that the 117 nucleotide stretch which derives from exon 5 is precisely deleted. Exons 6–8 are retained, and the reading frame is unaltered. The HLA-A24 transcript from which this cDNA derives would be expected to encode a molecule missing 39 amino acids, including the hydrophobic amino acids which would normally function to anchor the polypeptide in the plasma membrane, but retaining a normal carboxyl terminus. This agrees precisely with the structure proposed for the class I polypeptides secreted by HPB-ALL. These data thus provide strong evidence that alternative splicing of HLA-A24 transcripts does occur in this cell line, and that the resultant transcripts that lack exon 5 encode class I polypeptides that are secreted rather than membrane bound.

HLA-A and -B Antigens Are Both Secreted and Shed From a Variety of Other Cell Lines. The generality of these findings has been assessed by extending the immunochemical analysis to a variety of other cell lines. Supernatant-localized class I molecules could be detected in some, but not in all, cases. Certain cells displayed a particularly complex pattern of supernatant class I molecules, which required more careful analysis. An example of this is the B cell line AS (HLA-A2, -A24, -B27), for which data are presented in Figs. 7 and 8.

Fig. 7 A shows that AS culture supernatants contain three distinct size classes of class I polypeptides of 43,000, 39,000, and 35,000 D. TX-114 extraction shows that the 43,000-D form is detergent binding, while the 39,000- and 35,000-D forms are not (lanes 5–8). Ultracentrifugal flotation analysis was used to assess whether any of the species were lipid associated (Fig. 7B). All three species sediment through medium at a density of 1.06 g/ml (lanes 1–3). However, whereas the 39,000- and 35,000-D species sediment through medium at a density of 1.21 g/ml, the 43,000-D species floats (lanes 4–6). This indicates that the 43,000-D form is lipid associated, whereas the other forms are not. Hence, the 43,000-D polypeptides appear to be intact class I molecules which are shed from cells in association with membrane lipid. However, due to both the lack of detergent binding, and the absence of lipid association, the 39,000-D material cannot represent a proteolyzed form of the 43,000-D species. As argued previously, a 39,000-D proteolytic fragment of the heavy chain must contain the hydrophobic region, and as a result will bind TX-114 (Fig. 3A), and would be expected to be lipid associated. Thus the 39,000-D species detected here is likely to arise neither by proteolysis nor shedding, but rather by secretion as a result of an alternative splicing pathway, as described for HPB-ALL. The origin of the 35,000-D species is less certain, although it is reasonable to assume that it indeed may arise by proteolysis of the larger forms.

Both HLA-A and HLA-B products are found as lipid-associated 43,000-D species in AS culture supernatants (data not shown). Two-dimensional gel electrophoretic analysis of the water soluble fraction of AS culture supernatant is presented in Fig. 8. Whereas HLA-A2, -A24, and -B27 can all be detected in the cell lysate (Fig. 8, A–C), only HLA-A24 is present as a water soluble, 39,000-D form (Fig. 8, D–F). Hence, at least two distinct processes occur in this cell line: nonspecific shedding of lipid-associated molecules, and allele-specific secretion of water soluble class I molecules.
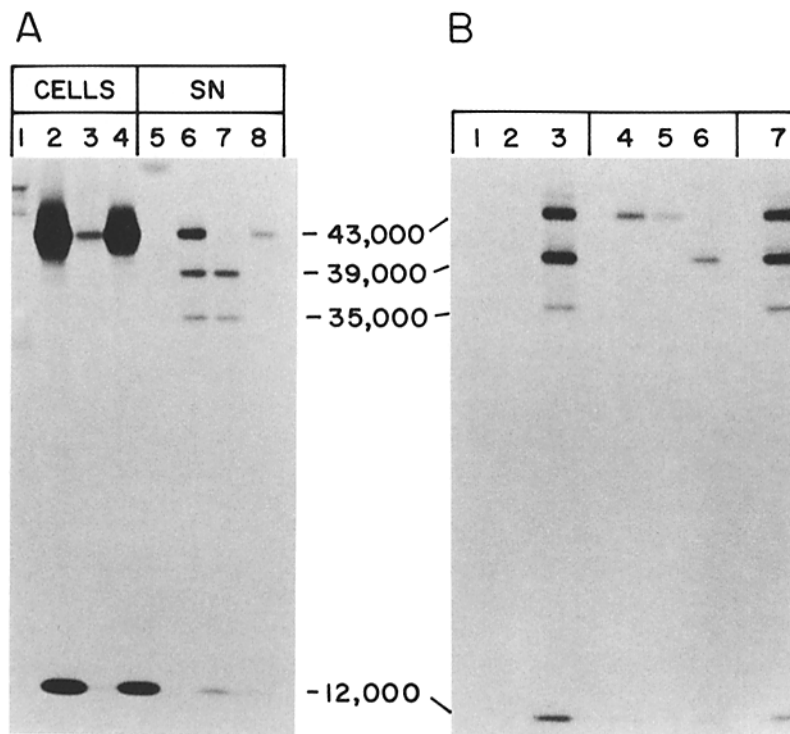
FIGURE 7.   Analysis of class I polypeptides in AS cell lysate and culture supernatant preparations. AS cells were labeled for 4 h in the presence of [^{35}S]methionine, after which culture supernatant was harvested, and cell lysates were prepared using either NP-40 or TX-114. (A) Polypeptides were immunoprecipitated from aliquots of NP-40 lysate using either normal rabbit serum (lane 1) or W6/32 (lane 2). An equivalent aliquot of TX-114 lysate was separated into aqueous (lane 3) and detergent phases (lane 4), which were then used for immunoprecipitation with W6/32. Polypeptides were immunoprecipitated from aliquots of culture supernatant using either normal rabbit serum (lane 5) or W6/32 (lane 6). TX-114 was added to an equivalent aliquot of supernatant, which was then separated into aqueous (lane 7) and detergent (lane 8) phases. Immunoprecipitation was then performed using W6/32. Aliquots of cell lysate represent 0.1 times the cell equivalents used for culture supernatant immunoprecipitations. (B) AS culture supernatant was brought to either 1.06 g/ml or 1.21 g/ml, after which ultracentrifugal flotation analysis was performed. Lanes 1–3 show the polypeptides immunoprecipitated from the upper, middle, and lower portions of the tube, respectively, following centrifugation at 1.06 g/ml. Lanes 4–6 show the polypeptides immunoprecipitated from the upper, middle, and lower portions of the tube, respectively, after centrifugation at 1.21 g/ml. Lane 7 displays the polypeptides immunoprecipitated from the culture supernatant before centrifugation. Immunoprecipitation was using W6/32, and analysis was by SDS-PAGE followed by fluorography.

This analysis was then extended to a panel of other cell lines. Secretion of water soluble, 39,000-D class I molecules was a property of some, but not all, cell lines. No cell line tested was found to express levels as high as HPB-ALL. Levels for some positive cell lines were as high as 1%, and for others were between 0.1 and 1%. If presumed intracellular precursors are included in the analysis, these levels would be doubled or tripled in most cases. These levels were independent of cell type, but seemed to vary, dependent upon HLA type. Specifically, the cell lines found to secrete at higher levels were typed as either

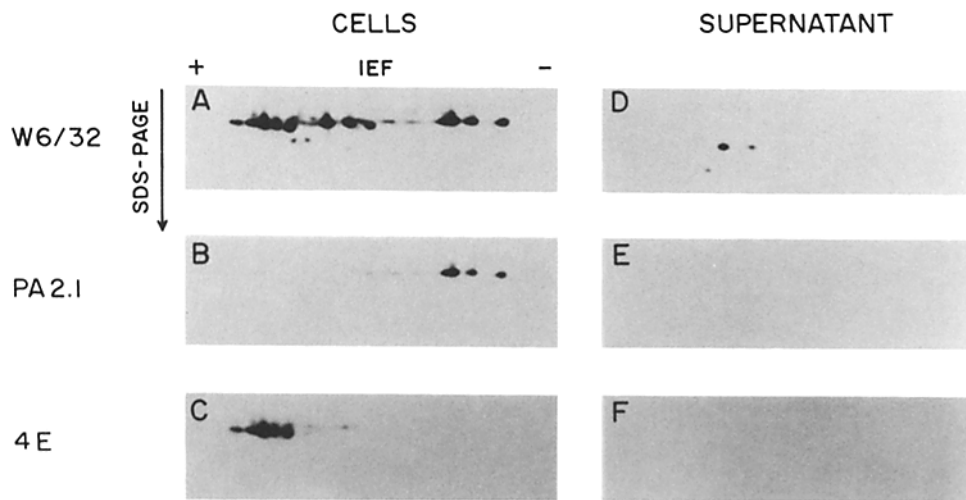CELLS   SUPERNATANT

FIGURE 8.   Two-dimensional gel electrophoresis of immunoprecipitates from AS cell lysate and culture supernatant aqueous phase. AS cells were labeled for 4 h in the presence of [^{35}S]methionine, after which cell lysate and culture supernatant were prepared. TX-114 was added to the culture supernatant, and the aqueous phase was used. Immunoprecipitation was with: W6/32 (HLA-A-, -B-, -C-specific), A and D; PA2.1 (HLA-A2-specific), B and E; and 4E (HLA-B-, -C-specific), C and F. Those spots immunoprecipitated by W6/32, but not immunoprecipitated by either PA2.1 or 4E are presumed to represent HLA-A24. Cell lysate immunoprecipitations were from 0.1 times the cell equivalents used for culture supernatant immunoprecipitations.

HLA-A24 or HLA-A9. These cell lines included the T cell line HPB-ALL (HLA-A24, -Bw51), the B cell lines AS (HLA-A2, -A24, -B27) and WT51 (HLA-A9, -B14), and the hepatoma cell line HEP-G2 (HLA-A2, -A9, -B5, -Bw17; reference 33). Based upon two-dimensional gel electrophoretic analysis, HLA-A9 or -A24 was the only allele secreted from AS and HEP-G2, and was the primary allele secreted from HPB-ALL and WT51. In fact, HLA-A24, along with HLA-A23, are recent splits of the broader HLA-A9 specificity, and thus all of these cell lines may secrete a related, if not identical, allelic product. Cell lines expressing intermediate levels of secreted class I molecules included the T cell lines Molt-3 (HLA-A1, -A10), 8402 (HLA-A1, -A29), and HSB (HLA-A1, -A2, -B12, -Bw17), as well as the B cell line SB (HLA-A1, -A2, -B12, -Bw17). Secretion was either extremely low or undetectable for the B cell lines MST (HLA-A3, -B7), JY (HLA-A2, -B7), 23.1 (HLA-A2, -B27), and T5-1 (HLA-A1, -A2, -B8, -B27).

HLA-A and -B Antigens Are Secreted by Normal PBL. Mitogen-activated PBL were examined for class I antigen secretion as well. Results for cells derived from three different donors, M.B. (HLA-A1, -A24, -B18, -Bw44), J.C. (HLA-A2, -A23, -B27, -Bw44), and M.L. (HLA-A2, -A11, -B7, -Bw35) are presented in Fig. 9, *A*, *B*, and *C*, respectively. In each case, 39,000-D class I polypeptides that partition into the TX-114 aqueous phase could be detected. At lower levels, 43,000-D polypeptides that partition into the TX-114 detergent phase could be detected as well. Levels of secreted, 39,000-D polypeptides were ~1% for M.B. and J.C. cells, but were lower for M.L. Two-dimensional gel electrophoretic analysis indicated that M.B. cells primarily secrete an HLA-A locus product, J.C.
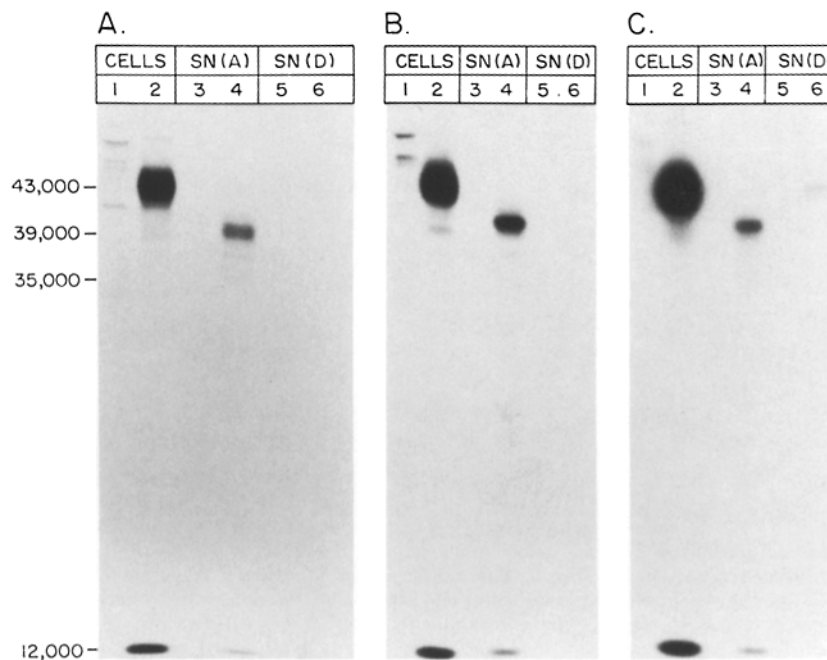
FIGURE 9.   Analysis of class I polypeptides in PBL cell lysate and culture supernatant preparations. PHA-stimulated PBL were labeled for 4 h in the presence of [^{35}S]methionine, after which culture supernatants (SN) were harvested, and detergent lysates (cells) prepared. TX-114 was added to culture supernatant preparations, which were then separated into aqueous (A) and detergent (D) phases. Immunoprecipitation was then performed using either normal rabbit serum (lanes 1, 3, and 5) or W6/32 (lanes 2, 4, and 6). Peripheral blood cells were from donors M.B. (A), J.C. (B), and M.L. (C). Cell lysate immunoprecipitations were from 0.1 times the cell equivalents used for culture supernatant immunoprecipitations. Analysis was by SDS-PAGE followed by fluorography.

cells primarily secrete an HLA-B locus product, and M.L. cells secrete HLA-A and -B locus products at comparable levels (data not shown). Thus secretion has been detected in both tumor cells and normal cells, and at the present level of analysis, occurs consistently, but not exclusively, for HLA-A24, and independent of cell type.

Discussion

Previous work has identified a mutant of the B lymphoblastoid cell line T5-1, called 8.14.1, which secretes a water soluble form of the HLA-A2 antigen (4, 5). Characterization of the secreted polypeptide indicated that it carries an internal deletion near the carboxyl terminus, including the hydrophobic amino acids that would normally function to anchor the polypeptide in the plasma membrane. It was proposed that this structure could result from an unusual RNA splicing event, which deletes exon 5 from HLA-A2–encoding transcripts. This was confirmed by the demonstration, by both Northern blot analysis and cDNA cloning, that transcripts precisely deleted for the 117 nucleotide stretch known to be encoded by exon 5 were present in the mutant, but not the parent cell line (6). Using these cell lines as a model system, a variety of approaches have been

developed, both at the protein and nucleic acid level, to assess whether a similar phenomenon occurs in nonmutagenized cells. The present work documents: (a) the secretion of class I molecules (primarily HLA-A24) by the T leukemic cell line HPB-ALL that have properties indistinguishable from those secreted by mutant 8.14.1; (b) alternative splicing that removes exon 5 from a fraction of HLA-A24-encoding transcripts; and (c) the secretion, possibly in an allele-specific fashion, of class I antigens from a variety of tumor cells and normal cells. This represents the first demonstration of bona fide, water soluble, secreted HLA antigens in nonmutagenized cells, and similarly the first demonstration of naturally occurring alternative splicing in the HLA system. It is not formally proven that in the case of HPB-ALL the unusually spliced HLA-A24 transcripts actually encode the HLA-A24 molecules secreted by this cell line. However the close correlation between the deduced structure and properties of the secreted molecules and the structure of the unusually spliced transcripts argues persuasively that this is so. This correlation is strengthened by the example of mutant 8.14.1, since it is distinguishable from the parent cell line by both the secretion of HLA-A2 and the unusual splicing of HLA-A2-encoding transcripts (5, 6).

Alternative splicing has been shown in a number of instances for murine class I antigens. Thus, it has been shown that the H-$2K^d$ gene gives rise to two transcripts that differ in the pattern of splicing at the 5' end of exon 2 (34), and the $Q10$ gene gives rise to transcripts that either include or exclude exon 3 (35). H-2D^d-encoding cDNAs have been obtained that either include or exclude exon 7 (36), and H-2K^q-encoding cDNAs have been obtained that result from the use of either an upstream or a downstream splice acceptor site at the 5' end of exon 8 (37). In those cases for which quantitative information is available, the non-standard splicing pattern appears to occur at a level ~5–10% that of the standard pattern (34, 35). Since the discovery of these splicing patterns has relied on the sequence analysis of a limited number of cDNA clones, it is very possible that other patterns of splicing may have gone undetected. That alternative splicing has not heretofore been documented for the HLA system is probably due to the more extensive analysis of murine cDNA clones to date. Whether these splicing patterns contribute to the functional heterogeneity of class I polypeptides remains to be determined. Unlike in some other systems, there is as yet no evidence for the regulation and/or tissue specificity of splicing. However, the total level of heterogeneity of class I antigen transcripts may be too high to attribute to mere sloppiness in the system.

Although soluble and/or serum forms of class I molecules have been reported previously, the mechanism described here is distinct. A nonpolymorphic Qa region gene, $Q10$, encodes a liver-specific, secreted class I polypeptide, which is detectable in murine serum (9–12). This molecule is apparently secreted because $Q10$ exon 5 contains numerous substitutions, a frameshift, and a termination codon, resulting in the synthesis of a truncated polypeptide lacking a true hydrophobic region. Similarly, it has been proposed that another gene within the Qa region, 27.1, might encode a secreted polypeptide, because it too carries a termination codon within exon 5 (35). On the other hand, Emerson et al. have shown that the products of the classical transplantation loci are shed, in association with membrane lipids, from the surface of radioiodinated or metabolically

labeled murine spleen cells (8). Intriguingly, this could be shown for H-2K^k, but not H-2D^k. This shedding process may be related to that which generates the lipid-associated, 43,000-D class I polypeptides found in culture supernatants of some of the cell lines examined here, including AS. However, no evidence has been accumulated to suggest any allelic specificity in the cell lines examined.

Using alloantisera, and more recently mAbs, it has been possible to detect HLA-A and -B specificities in 100,000 g supernatants of human serum (reviewed in reference 7). The precise structure and origin of these molecules is unclear. However, it appears that different allelic products are present in serum at reproducible and characteristic levels. Notably, HLA-A9 has been reported to be present at levels significantly higher than any other allele tested (7, 38). This is particularly striking in view of the results reported here, since HLA-A9 and HLA-A24 (a recent serological split of the HLA-A9 specificity) appear to be secreted in water soluble form at levels higher than the product of any other allele examined. Paradoxically, the HLA-A9 in human serum has been reported to be lipoprotein-like, based upon ultracentrifugal flotation under conditions identical to those used in this report (21). Whether these disparate observations can be reconciled awaits further analysis.

Our present understanding of the function of class I molecules is limited to the cell surface forms of the products of the classical transplantation loci. In this form they appear to mediate interactions between CTL and virally infected target cells. However, functions have yet to be ascribed to even the cell surface forms of the Qa and Tla region gene products. There is at present no good conceptual framework that accommodates the presence of soluble forms of any class I molecule. However it now appears that a number of distinct forms of such molecules clearly exist. Whether they serve an important functional role, or are in any other way of physiological consequence, remains to be determined.

Summary

Human class I major histocompatibility antigens (HLA-A, -B and -C) are integral membrane protein heterodimers, which are anchored in the membrane via a stretch of hydrophobic amino acids near the carboxyl terminus of the heavy chain. It has previously been shown that a mutagenized cell line secretes a water soluble form of the HLA-A2 antigen, due to a pattern of RNA splicing that removes exon 5 (encoding the transmembrane hydrophobic amino acids) from mature, HLA-A2–encoding transcripts. The present study was undertaken to assess whether a similar process might be operative in nonmutagenized cells. It is shown that water soluble class I molecules (primarily HLA-A24) are secreted by the T leukemic cell line HPB-ALL, and that alternative splicing removes exon 5 from a fraction of HLA-A24–encoding transcripts. It is further shown that class I molecules are secreted, possibly in an allele-specific fashion, from a variety of tumor cells and normal cells. The possible relationship between these findings and previous reports of HLA-A and -B antigens in human serum is discussed.

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