

Embryonal carcinoma cells express Qa and Tla class I genes of the major histocompatibility complex

(transplantation antigens/development/teratocarcinoma/tumor recognition)

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ABSTRACT The murine major histocompatibility complex encodes H-2K and H-2D transplantation antigens and other class I-like proteins called Qa and Tla molecules; the functions of the Qa/Tla molecules are not known. That they may participate in embryonic cell–cell interactions and/or play a role in immune responses against tumors has been speculated. We have studied two murine embryonal carcinoma tumors, 402AX and PCC4, that are rejected *in vivo* immunologically, although they do not express H-2K or H-2D antigens. Transplantation studies with these cells suggest that rejection is mediated by class-I-like major histocompatibility complex antigens. As a first step in evaluating Qa/Tla function(s), we have characterized expression of class I-like genes and proteins in 402AX and PCC4 cells. Northern (RNA) blot hybridizations, polymerase chain reaction studies, and cDNA cloning experiments demonstrate that EC lines transcribe genes allelic to the Tla region gene “37”, Qa-2 region gene “Q7”, and another, previously uncharacterized, class I-like gene. Immunoprecipitation studies show that the embryonal carcinoma tumor cells contain low levels of β_2 -microglobulin expressed in association with non-H-2K, non-H-2D class I-like proteins.

The murine major histocompatibility complex (MHC) encodes cell-surface glycoproteins involved in immune-mediated recognition of foreign and self antigens. These molecules include the well-characterized and highly polymorphic class I transplantation antigens. The MHC also encodes the less polymorphic Qa and Tla class I antigens. Although these molecules are similar in structure to the class I antigens and associate with β_2 -microglobulin (β_2m), their function is unknown. Because the H-2, Qa, and Tla antigens are differentially expressed during early development (1, 2), it has been hypothesized that they participate in embryonic cell–cell interactions. This hypothesis is supported by the observation that the gene encoding the Qa-2 antigen may determine the rate of cleavage of cells in the preimplantation embryo (3). Another hypothesis states that Qa and/or Tla antigens may serve as restriction elements recognized by the subset of T lymphocytes expressing the T-cell receptor γ - and δ -chain complex (4–6). Other studies (7–12) lead to the speculation that class I-like antigens may function in presentation of tumor antigens to T lymphocytes. This may be particularly valid for tumor antigens of embryonic origin that are not expressed during the T-cell tolerance induction stage but reoccur later in life on malignant cells. Nonpolymorphic Qa/Tla antigens could have, hypothetically, evolved to serve as their restriction elements.

We are using murine teratocarcinomas as a model system in which to study the function of Qa/Tla class I-like antigens.

These tumors include malignant, undifferentiated embryonal carcinoma (EC) stem cells that do not express serologically detectable H-2K and H-2D antigens. Transplantation and cellular immunity data suggest, however, that EC cells express other class I-like molecules that may function as restriction elements and/or transplantation antigens in anti-tumor immunity (7–9). Because of their developmental pluripotency and ability to differentiate *in vitro*, EC cells may also be an experimental system in which to study the role of classical (H-2K and H-2D) and nonclassical (Qa and Tla) class I antigens in differentiation and development.

In the present studies we have characterized the expression of class I-like proteins and H-2 homologous transcripts in two different EC cell lines. We find that both EC cell lines express class I-like molecules and transcribe Qa and/or Tla genes. These results are discussed in the context of the differences between H-2K, H-2D, and Qa/Tla expression.

MATERIALS AND METHODS

Materials. Mice were purchased from The Jackson Laboratory. The 402AX cell line was maintained as described (13). The PCC4 cell line was grown in minimal essential medium/10% calf serum/1% L-glutamine (GIBCO) under sparse culture conditions ($< 1 \times 10^3$ cells per ml) to maintain them in their undifferentiated state. Monoclonal antibodies (mAbs) were prepared as described (14).

Immunoprecipitations. Cells were labeled with [³⁵S]methionine, and immunoprecipitations were performed as described (15).

cDNA Libraries. cDNA libraries were prepared as described (16–18) with modifications suggested by H. Cheroutre (University of California, Los Angeles). Total RNA was isolated from 10^9 402AX and PCC4 cells (19). Poly(A)⁺ RNA was prepared on Hybond-mAP paper as recommended by the manufacturer (Amersham). cDNA was synthesized from 3 μ g of poly(A)⁺ RNA and size selected on an agarose A1.5 column (0.5 \times 10 cm). Largest cDNAs [> 1.5 kilobases (kb)] were ligated into the *Eco*RI site of λ gt10. Recombinant phage were packaged and plated without amplification.

DNA Probes and Library Screening. The AC-14 probe contains a 439-base *Alu* I fragment of nucleotides 2540–2979 of the *H-2D^d* gene cloned into the *Sma* I site of m13mp8 vector (20); pH2IIa was described (21).

cDNA libraries were screened with a *Bam*HI–*Hind*III AC-14 fragment isolated from m13mp8 vector and rescreened with pH2IIa.

DNA Sequencing. DNA sequencing was performed by the dideoxy chain-termination method (22).

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Abbreviations: EC, embryonal carcinoma; MHC, major histocompatibility complex; β_2m , β_2 -microglobulin; mAb, monoclonal antibody; TM, transmembrane; PCR, polymerase chain reaction.

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Northern (RNA) Blot Analysis. Total RNA was isolated (23), electrophoresed, blotted, and hybridized with class I or actin probes. Class I pH2IIa-EH probe was constructed by Elly Holthuizen (California Institute of Technology). This probe contains a 276-base-pair (bp) *Pst* I-*Sac* I fragment of pH2IIa α 3 coding region cloned into Bluescript KS-2 vector. Actin probe was constructed by subcloning a \approx 900-bp *Pst* I fragment of mouse α -skeletal actin cDNA into Bluescript vector. The actin cDNA was cloned by S. B. Sharp (California Institute of Technology) from a library constructed by R. J. LaPolla and N. Davidson (California Institute of Technology). Hybridizations were at 37°C in 5 \times SSC (1 \times SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7) for 12–18 hr. Filters were washed three times in 0.1% SDS with 2 \times SSC, 1 \times SSC, and 0.2 \times SSC at 65°C.

Polymerase Chain Reaction (PCR). Two micrograms of total RNA from cell lines and spleen cells (23) was converted to cDNA using an Amersham cDNA synthesis kit. PCR reactions (35 cycles) were done as in ref. 24 by using as primers oligonucleotide TTGCCCACTTGCTTCAGCTGCTGGTCAG from exon 1 and oligonucleotide CTTCTGAGGCCAGTGAGAGGTCTG from exon 7 of the “37” gene. Amplification products were hybridized for 16 hr at 37°C in 6 \times SSC/1% SDS/denatured salmon sperm DNA at 100 μ g/ml/0.5% nonfat milk/10% (wt/vol) dextran sulfate containing a “37” gene-specific transmembrane probe (CCTATGTGTCTCCTCC). The specificity of the probe was verified by hybridization with a panel of BALB/c class I genes. Membranes were washed in hybridization fluid without probe at 45°C for 15 min, in 0.1 \times SSC/1% SDS at room temperature for 15 min, and in 3.2 M tetramethylammonium chloride at 50°C for 10 min.

RESULTS

EC Cells Express β 2m-Associated Class I-Like Proteins. In the present study we have analyzed two independently isolated EC cell lines. The 402AX line was originally derived from a primary spontaneous testicular teratocarcinoma of a 129/Sv (*H-2^b*, *Tla^f*) mouse (25) and has been adapted to cell culture. This cell line does not express classical H-2K or H-2D antigens and does not differentiate *in vitro*, even in the presence of differentiating agents. Immune rejection of 402AX cells involves the induction of tumor antigens serologically detected by H-2K and H-2D antibodies (14). *In vitro* studies suggest that other class I-like molecules also function in T-cell mediated recognition of 402AX cells (7).

PCC4 is a culture-adapted cell line derived from the 129/Sv strain OTT6050 teratocarcinoma (26). Under low-density culture conditions PCC4 does not differentiate and does not express H-2K or H-2D antigens. When cultured at higher densities or upon treatment with differentiation inducers, PCC4 differentiates morphologically and biochemically and expresses H-2K and H-2D antigens (27). The PCC4 line has not been extensively studied *in vivo*, but growth of its sister clone PCC3, derived from the same parental tumor, has been analyzed (8). Rejection of the PCC3 tumor occurs in the absence of serologically detectable H-2K and H-2D expression. Transplantation analyses suggest, however, that the rejected PCC3 cells express antigens, which at the T-cell epitope level, are closely related to H-2K and H-2D antigens.

To investigate whether EC cells express biochemically detectable class I-like molecules, we have carried out immunoprecipitations on whole-cell lysates. We reasoned that EC class I-like molecules may be encoded by *Qa* and/or *Tla* genes. Available antibodies, however, only detect the gene products of a very few of the \approx 35 known *Qa* and *Tla* genes. Because most class-I antigens are noncovalently associated with the highly conserved β 2m chain, we used a rabbit anti-mouse- β 2m antiserum to immunoprecipitate 402AX and

PCC4 class I-like molecules. This strategy has been used previously to precipitate and identify the nonclassical *Q4* gene product (28, 29).

Two products are immunoprecipitated with the anti- β 2m antiserum from 402AX and PCC4 extracts (Fig. 1, lanes e and i): a <14-kDa protein that comigrates with 129/J splenocyte β 2m (lane a) and 44- to 48-kDa proteins that migrate within the molecular mass range predicted for class I polypeptides. The anti-H-2K^b (20-8-4) and anti-H-2D^b (28-14-8) mAbs immunoprecipitate H-2K and H-2D antigens (lanes b and c) from control 129/J splenocytes but are not reactive with 402AX and PCC4 cells (lanes f, g, j, and k). Negative control anti-H-2K^k antibodies (11-4-1) do not react specifically with any of the cell extracts (lanes d, h, and l). The faint band of \approx 43 kDa in the 11-4-1 lanes corresponds in molecular mass to actin, and is also seen in control bovine serum albumin-treated lysates (data not shown). Because it has been previously reported that other EC cells do not transcribe β 2m genes (30, 31), we determined the level of β 2m polypeptide expression.

That H-2D^b antigen can be expressed on the cell surface without β 2m has been shown (32). Therefore, 402AX cells were transfected with the *H-2D^b* gene fused to a Moloney murine leukemia virus promoter (33), and the transfectant clones positive with anti-H-2D^b 28-14-8 mAb were biosynthetically labeled and immunoprecipitated with 28-14-8 mAbs. High levels of H-2D^b heavy chains in these transfectants coprecipitated with trace amounts of β 2m (data not shown). In contrast, H-2D^b antigens from activated 129/J splenocytes precipitated with high levels of β 2m chains (Fig. 1, lane c). Quantitative densitometry tracings of the two autoradiographs indicate that the level of β 2m coprecipitated with H-2D^b antigen in 402AX transfectants is < \approx 6% of the β 2m coprecipitated with H-2D^b antigens in 129/J splenocytes (data not shown).

EC cells, therefore, express very low levels of β 2m available for association with H-2 class I heavy chains. The β 2m proteins found in 402AX and PCC4 cells are associated with non-H-2 class I-like polypeptides.

EC Cells Express MHC Class I Transcripts. Earlier studies demonstrated that EC cells do not transcribe mature H-2 mRNAs (30, 31). These experiments relied on Northern (RNA) analyses of EC RNA with H-2 class I probes and may not have been sensitive enough to detect very low levels of heterogeneous species of class I-like transcripts, especially if

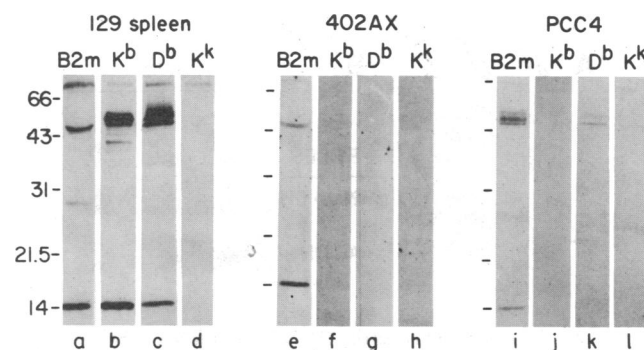


FIG. 1. Immunoprecipitations of MHC class I antigens from 129/J, 402AX, and PCC4 cells. Cell extracts containing approximately equal radioactivity were immunoprecipitated with each antibody. 129/J Con A-stimulated blast (lanes a–d), 402AX (lanes e–h), and PCC4 (lanes i–l) cells were immunoprecipitated with a rabbit anti-mouse- β 2m polyclonal antibody (β 2m; lanes a, e, and i), 20-8-4 mAb (anti-K^b; lanes b, f, and j), 28-14-8 mAb (anti-D^b; lanes c, g, and k), or 11-4-1 mAb (anti-K^k; lanes d, h, and l). Molecular mass markers are bovine serum albumin (66 kDa), ovalbumin (43 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa).

these transcripts had low homology to the probes. Because the size, nucleotide sequence, and relative abundance of each of the *Qa* and *Tla* transcripts are not known, we have sought to identify class I-like genes expressed in EC cells by three methods.

Northern analysis of EC transcripts was performed using as a probe pH2IIa-EH, which is homologous to the conserved $\alpha 3$ coding region of an unidentified class I-like gene and hybridizes with all known BALB/c *Qa* and *Tla* genes as well as with *H-2K^d* and *H-2D^d* genes. PCC4 and 402AX cells express low but detectable levels of mature ≈ 1.7 -kb class I mRNAs (Fig. 2A). Densitometry quantitations of the EC class I RNA bands and control actin RNA bands (see Fig. 2B) indicate that PCC4 cells express $\approx 2\%$ and 402AX cells express $\approx 7\%$ of the class I RNA expressed in C57BL/6 spleen cells.

To identify the class I RNAs present in EC cells, 402AX and PCC4 cDNA expression libraries were prepared. Because immunoprecipitation data demonstrate that 402AX and PCC4 cells express class I-like molecules that associate with $\beta 2m$, we screened the libraries with the AC-14 and pH2IIa probes derived from the fourth exon of H-2 genes. The screening yielded 2 positive plaques of 8.5×10^5 total plaques in the 402AX library and 136 positive plaques of 1.8×10^6 total plaques in the PCC4 library. DNAs from positive clones were digested with *EcoRI* and sized by Southern blotting. The positive clones fell into four classes: 402AX 2.4-kb, PCC4 1.5-kb, PCC4 2.5-kb, and PCC4 3.0-kb clones. Within a given group all cDNA inserts gave identical restriction enzyme patterns when tested with *Pvu* II, *Pst* I, *Rsa* I, *Hind* III, *Xba* I, and *Xho* I. The clones within each group also hybridized with the same probes when analyzed with class I gene-specific DNA fragments and oligonucleotides (data not shown). A representative clone from each group was partially sequenced and compared with known class I genes of BALB/c (*Tla^a*) and C57BL/10 (*Tla^b*) mice. Fig. 3 shows the sequencing strategy and aligns the four cDNA clones with the H-2 genes most homologous with them. All of the clones differ from classical H-2 genes and contain regions, which in classical H-2 transcripts, correspond to introns.

The 402AX and two of the PCC4 cDNA clones correspond to known *Qa*- or *Tla*-region genes. The PCC4 1.5-kb clone (Fig. 3A) is almost identical in the sequenced regions to the *Qa-2* region gene, *Q7* (21, 36). The only variant nucleotide between PCC4 1.5-kb clone and BALB/c *Q7* sequence is

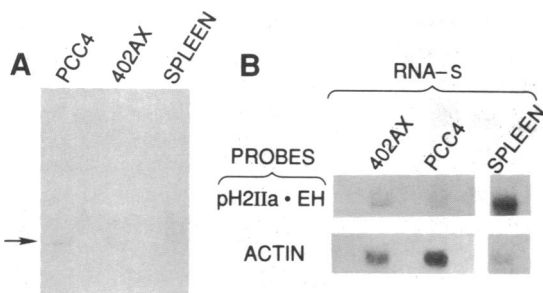


Fig. 2. Northern analyses of MHC class-I mRNA in EC cells. (A) Detection of class I mRNAs. Approximately 20–40 μ g of EC RNA and ≈ 0.2 μ g of C57BL/6 spleen RNA was loaded per lane. Northern hybridizations were done by using pH2IIa-EH as a class I-exon 4-specific probe. All hybridizing bands have the same apparent mobility relative to 18S and 28S rRNA. The visible difference in class I band migration between EC and spleen lanes is caused by the ≈ 100 -fold difference in quantities of RNAs loaded. (B) Quantitation of class I RNAs. Two identical nylon filters carrying blotted 402AX, PCC4, and C57BL/6 spleen RNAs were hybridized with labeled pH2IIa-EH and actin probes and autoradiographed. The bands were scanned by densitometer, and the level of class I mRNA was estimated relative to the level of actin mRNA.

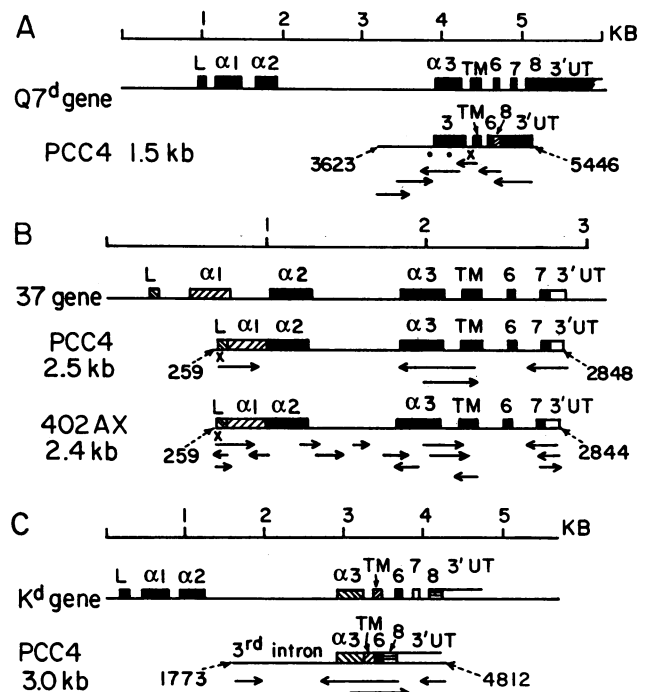


Fig. 3. Sequencing strategy and comparison of 402AX and PCC4 cDNA clones to known MHC class I genes. UT, untranslated region. (A) Comparison of PCC4 1.5-kb clone to BALB/c *Q7^d* gene (ref. 21; *Q7^d* corrected GenBank sequence entered by D.N.). Dots under the third intron and fourth exon correspond to positions 3923 and 4085, where the PCC4 1.5-kb clone is adenine and cytosine as is *Q7^d*, whereas the *Q7^b* allele is guanine and thymine. X under the fourth intron denotes the only polymorphism between the PCC4 1.5-kb clone and *Q7^d*. It is an adenine at position 4246 instead of the guanine found in the published *Q7^d* sequence. Numbering is according to the corrected GenBank *Q7^d* (27.1) sequence. (B) Comparison of PCC4 2.5-kb and 402AX 2.4-kb clones to the *Tla* region "37" gene (34). PCC4 2.5-kb and 402AX 2.4-kb sequences are identical with the "37" gene sequence, except for three bases 5' to the ATG codon (see Fig. 4) within the first exon (marked with X). The PCC4 2.5-kb clone extends four bases beyond the 402AX 2.4-kb clone at the 3' end. (C) Comparison of PCC4 3.0-kb clone to *K^d* gene (35). PCC4 3.0-kb clone is 80–85% homologous to *K^d* within intron 3 and exons 5–8 and is $\approx 40\%$ homologous in the 3'-untranslated region. Solid arrows indicate sequencing runs. Broken arrows indicate beginning and ending of cDNA clones. $\alpha 1$, $\alpha 2$, and $\alpha 3$ designations refer to the exons encoding the three external domains, TM is the transmembrane domain, and exons 6–8 encode cytoplasmic domains of MHC class I genes.

located at position 4246 in the fourth intron. PCC4 1.5-kb differs from C57BL/10 *Q7* sequence by two nucleotides located at positions 3923 and 4085. These substitutions presumably correspond to allelic differences between 129/Sv, C57BL/10, and BALB/c genomes. The PCC4 2.5-kb clone and the 402AX 2.4-kb clone are almost identical to each other and are 98–100% homologous to the BALB/c *Tla* region "37" gene (ref. 34; Fig. 3B). The only differences between the "37" gene from a DBA/2 library and the EC clones are located in the 5' untranslated region just upstream from the ATG codon of exon 1 (Fig. 4A). The 402AX 2.4-kb and the PCC4 2.5-kb clones differ from each other only in their length at the 3' end, where the PCC4 2.5-kb cDNA is four bases longer than the 402AX 2.4-kb clone (Fig. 3B). The PCC4 3.0-kb cDNA clone does not correspond to any known sequenced class I gene, although it is highly homologous to H-2 genes, particularly in the $\alpha 3$ and TM regions where it is 80–90% homologous to several *Qa*- and *Tla*-region genes (Fig. 3C and 4B).

Because cDNA clones from EC cells contained introns, we used the PCR technique to determine whether EC cells

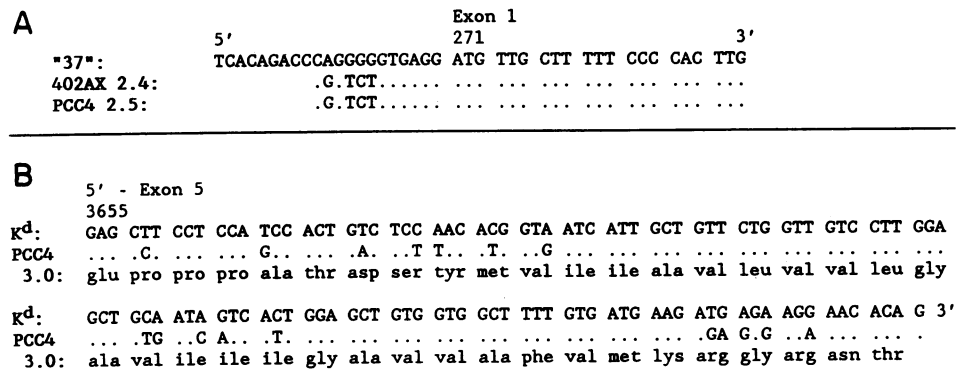


FIG. 4. Partial nucleotide sequences of 402AX 2.4-kb and PCC4 2.5-kb cDNA clones and partial nucleotide sequence and deduced amino acid sequence of PCC4 3.0-kb cDNA clone. The 402AX 2.4-kb and PCC4 2.5-kb clones (A) are compared with the Tla region DBA/2 "37" gene in the 5' untranslated region showing the four bases that differ between the EC clones and the "37" gene. The PCC4 3.0-kb cDNA (B) is compared with the K^d gene for the TM region. A dot at a position indicates identity with the compared sequence.

express fully spliced species of the class I transcripts. Oligonucleotides corresponding to the 5' and 3' ends of the "37" gene were used as primers for the PCR. The presence of the RNA species was monitored by Southern hybridization of PCR bands. Hybridization of the amplified PCC4 cDNA with the radiolabeled "37"-specific oligonucleotide shows two bands (Fig. 5). One band corresponds in size to the partially spliced PCC4 2.5-kb cDNA and the second band to the fully processed "37" transcript.

Thus, PCC4 cells transcribe both spliced and unspliced "37" mRNAs, whereas spleen RNA shows expression of fully processed "37" transcripts only.

DISCUSSION

EC cells have been used experimentally as a model system for cellular differentiation, preimplantation development (37), and tumorigenesis (38). Because of the critical role of MHC class I antigens in lymphoid cell recognition and their potential role in cell-cell interactions during embryogenesis, it is important to determine whether class I molecules are expressed in EC cells. We have studied the expression of class I molecules and transcripts in two independently derived EC cell lines. Immunoprecipitation studies and analyses of cDNA libraries demonstrate that 402AX and PCC4 cells express low levels of nonclassical MHC products. Based on DNA sequence analysis, both cell lines transcribe the Tla region "37" gene, which has been proposed to encode an ~45-kDa nonpolymorphic cell-surface molecule (10, 39). The "37" gene is transcribed in a wide variety of cell types and tissues, including H-2K⁻, H-2D⁻ brain (10) and 12- to

18-day fetal liver (34). "37" gene transcripts have been reported not to accumulate in F9 EC cells (34); this result may reflect differences between F9 and 402AX and PCC4 cells. Alternatively, the RNase mapping experiments on F9 cells may have been less sensitive in detecting heterogeneous species of rare "37" transcripts than the cDNA analyses and PCR experiments on 402AX and PCC4 cells.

In addition to expressing the "37" gene transcript, PCC4 cells express two other class I transcripts. One of them has been identified by DNA sequence analysis as the product of the Q7 gene. The Q7 gene encodes Qa-2 antigens detected on lymphocytes. These antigens are ~40-kDa proteins that are either attached to the plasma cell membrane by means of a phospholipid tail (33, 40) or secreted (40). The Q7 gene displays a complex pattern of expression during development. Serological studies suggest the presence of Qa-2 antigens on two-cell-stage mouse embryos (3). Classical H-2 antigens are not detectable until approximately day 11 of development (1). RNA studies support the serological results and detect Q7 mRNA in 8.5-day embryos (2). Our identification of Q7 transcripts in PCC4 EC cells is consistent with reports of Q7 expression early in development and further demonstrates similarities between EC cells and early embryonic cells.

The PCC4 3.0-kb clone represents an unidentified class I gene; its sequence differs from the sequence of all known class I genes.

All of the isolated EC class I cDNAs correspond to nonclassical class I products. This finding is consistent with previous reports showing very low levels or lack of H-2K or H-2D transcription in EC cells (30, 31) and in early embryos (41). Our results suggest that Qa/Tla gene transcription is regulated in EC cells independently of H-2K and H-2D expression. In contrast to the classical H-2 antigens, nucleotide sequences of the regions involved in the transcription initiation of the Q7 and "37" genes lack conventional TATA boxes. All clones identified in the 402AX and PCC4 libraries carry some intron sequences. In other studies (42), simian virus 40-infected EC cells were noted to contain nonspliced early RNA transcripts. Therefore, it has been proposed that EC cells regulate expression of certain genes by a posttranscriptional mechanism that involves "turning off" of the specific mRNA-splicing events (42). Qa and Tla transcripts in EC cells may be partially regulated by a similar mechanism. If so, then incompletely spliced Qa and Tla transcripts would coexist with mature, fully spliced RNAs in EC cells. The PCR experiments demonstrate the presence of unspliced, as well as mature, "37" mRNAs in PCC4 cells, even though Northern analysis failed to detect accumulation of unspliced RNAs. This result may be explained by differences in specificity of the probes used in these two approaches. The absence of fully

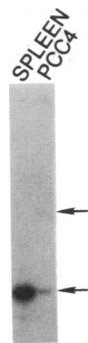


FIG. 5. PCR on cDNAs synthesized from C57BL/6 spleen and PCC4 RNAs. The predicted size of the PCR band for fully spliced "37" mRNA is ~1050 bases and for the unspliced band is ~2500 bases. The arrows point to PCR bands corresponding to the presumed fully spliced and unspliced "37" transcripts. A 1-kb λ ladder was used as a size marker.

spliced class I cDNAs is probably the result of the size selection and/or self-priming second-strand synthesis process used in preparation of the cDNAs. Independent of these considerations, the data are unambiguous in showing transcription of *Qa* and *Tla* genes in EC cells.

Immunoprecipitation studies demonstrate that 402AX and PCC4 cells express low levels of class I-like molecules associated with $\beta 2m$. The frequency of class I transcripts in 402AX and PCC4 RNA and in the cDNA libraries correlates with low-level expression of these class I proteins. It is unclear whether the class I proteins seen in EC cells are direct products of the *Qa* and/or *Tla* genes identified by cDNA cloning. More sensitive techniques may be necessary to identify all transcriptionally active class I genes in PCC4 and 402AX cells and to detect their protein products.

It has recently been hypothesized that T cells expressing the T-cell antigen receptor γ and δ chains recognize nonclassical MHC class I antigens (4–6). This hypothesis suggests that the *Qa* and/or *Tla* gene products identified in this study may serve as the target structure(s) for T-cell recognition of the 402AX and/or PCC4 cells. Additional studies are necessary to determine whether “37”, Q7, or other class I molecules are present in sufficient quantity on the cell surface of EC cells and whether these class I genes encode antigens involved in T-cell-mediated antitumor immunity.

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