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MHC class II-deficient tumor cell lines with a defective expression of the class II transactivator

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Abstract

MHC class II expression defects have been evidenced in several human tumor cell lines originating from lung cancers or retinoblastoma. Accordingly, the mouse adenocarcinoma and fibrosarcoma cell lines, RAG and L(tk$^-$), do not express I-A and I-E molecules even when treated with IFN-γ. Here we show that fusion of both cell lines restores the inducible expression of MHC class II, thereby demonstrating that they present different and recessive alterations outside the MHC class II locus. CIITA, the MHC class II transactivator, controls the tissue-specific expression of MHC class II genes and creates the architecture of the transcriptional complex that binds to the MHC class II gene promoters. In L(tk$^-$) cells, *C2ta* transcripts, expressed from the gene encoding CIITA, were indeed detected in severely limited amounts, with a defect in *C2ta* transcription initiation. In agreement we show here that the L(tk$^-$) cell line does not express the CIITA protein. In contrast, in the RAG cell line, *C2ta* transcripts were expressed at normal levels, from the proper initiation site. The nucleotide sequencing of the CIITA cDNA from RAG did not reveal any mutation. However, the CIITA protein was not detected. These data evidence a new type of defect in a MHC class II-defective tumor cell line, as we show here that the alteration in the RAG cells occurs downstream of *C2ta* transcription. The RAG mutation might therefore reside in the *C2ta* transcript nuclear export or translation, or in the stability of the CIITA protein.

Introduction

MHC class II molecules play a central role in the presentation of antigens to CD4$^+$ T lymphocytes during the initiation of the immune response (1). Constitutive expression of MHC class II molecules is restricted to professional antigen-presenting cells (APC) including B lymphocytes, dendritic cells and activated macrophages. Non-professional APC like endothelial cells, astrocytes, epithelial cells or fibroblasts can be induced to express MHC class II by IFN-γ (2). MHC class II gene expression is mainly regulated at the transcriptional level by four conserved *cis*-acting promoter elements termed W, X1, X2 and Y boxes (2,3).

Genetic complementation analysis carried out with cell lines derived from patients affected by MHC class II deficiency (4) defined four complementation groups (groups A–D) (5). Cell lines from complementation groups B, C and D display mutations in RFXANK (6), RFX5 (7) and RFXAP (8) respect-

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ively. These proteins correspond to the three subunits of the RFX transcription factor that binds to the X1 motif of the MHC class II gene promoters. Group A patients present mutations in the class II transactivator (CIITA) (9). This non-DNA-binding factor interacts with different transcription factors [regulatory factor X (RFX), nuclear factor Y (NFY) and cAMP-responsive element binding protein (CREB)] (10), the CREB-binding protein (CBP) transactivator (11) and the transcription machinery (12,13). Through these multiple interactions CIITA creates the transcriptional scaffold that permits the initiation of MHC class II gene transcription (14). In addition, CIITA is considered to be the master regulator of MHC class II expression and it has been demonstrated through mutant cell lines that it mediates the IFN-γ induction of MHC class II molecules (15). Indeed the tissue-specific pattern of expression of the MHC class II genes is controlled by CIITA, the expression of which precedes that of MHC class II (16,17). Cell- and tissue-specific patterns of *MHC2TA* or *C2ta* (the human and mouse genes respectively) expression are controlled by the alternative usage of three distinct promoters. Promoters I and III regulate the constitutive expression of CIITA in dendritic cells and B lymphocytes respectively, whereas promoter IV drives the IFN-γ-induced transcription of CIITA (18,19).

Modulation of MHC class II expression has been evidenced during the development of different types of tumors (20). The role of MHC class II deregulation in tumor cells is not clearly understood. It has been shown that the re-expression of MHC class II molecules in mouse sarcoma cells favors tumor regression (21). In contrast, in patients with melanoma tumors the abnormal constitutive expression of HLA-DR in melanoma cells has been associated with a bad prognosis (22). One main common point to the MHC class II deregulation is that CIITA expression is usually affected. Indeed, the analysis of the molecular events leading to MHC class II deficiency in human breast cancer and in non-small lung cancer cell lines has shown evidence of defects in *MHC2TA* transcription (23). In addition we have demonstrated the constitutive transcription of *MHC2TA* abnormally initiating from promoter III in melanoma cell lines constitutively expressing the MHC class II molecules (24).

Accordingly, here we described two additional tumor cell lines defective for expression of CIITA and explore the molecular mechanisms responsible of their defects. We show that in one cell line, RAG, transcription of *C2ta* is normal, while CIITA protein cannot be detected in these cells, suggesting that they display a defect in *C2ta* mRNA processing or protein stability. In contrast, we find that the second cell line, L(tk$^-$), expresses an aberrant form of the *C2ta* transcript.

Methods

Cell lines and IFN

RAG (a mouse adenocarcinoma cell line), L(tk$^-$) (a mouse fibrosarcoma cell line), WISH (a human amniotic cell line), RAW264.7 (a mouse monocyte-macrophage cell line), J777.4 (a mouse pre-monocyte cell line) and A20 (a mouse B cell lymphoma) were obtained from ATCC (Rockville, MD). The LM and GES human B cell lines were immortalized with Epstein–Barr virus. The RAW264.7 and WISH cell lines express MHC class II molecules after IFN-γ induction. The LM and GES cell lines constitutively express MHC class II molecules. The R.GES.2.1 cell hybrid originates from the fusion of the RAG and GES cell lines, and contains different human chromosomes including chromosome 16 (25). The RR2.16 cell line is a radiation somatic cell hybrid prepared from the R.2417.2 somatic cell hybrid (25). The resulting RR2.16 hybrid presents the genetic background of the RAG cell line and contains fragments of human chromosome 16 (our unpublished data). The RAG, J777.4, R.GES.2.1, RR2.16 and L(tk$^-$) cell lines were grown in DMEM supplemented with 10% FCS, antibiotics and 2 mM glutamine. The A20, RAW264.7, WISH, GES and LM cells were maintained in RPMI 1640 supplemented with 10% FCS, antibiotics and 2 mM glutamine under standard conditions. The specific activity of the mouse IFN-γ (kindly provided by Roussel-Uclaf, Romainville, France) was estimated by antiviral assay as previously described (26) and it was used at 150 or 250 U/ml as indicated in the figure legends.

Flow cytometric analyses

Subconfluent cell cultures were treated for 72 h with IFN-γ. Indirect immunofluorescence assays were performed using the following primary mAb: 20.8.4 (anti-H-2^d) (27), MKD6 (anti-I-A^d) (28), 11.4.1 (anti-H-2^k; ATCC TIB 95) and H39.64.5 (anti-I-A^k) (28). Cells were next labeled with FITC-conjugated sheep anti-mouse Ig from Biosys (Compiègne, France). Flow cytometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA) using the Lysys II program (Becton Dickinson).

Cell fusions

Somatic cell hybrids were prepared by the fusion of 10^7 cells of each fusion partner using PEG 4000 (Merck, Darmstadt, Germany) following previously described protocols (29). Adherent hybrids generated by the fusion of the L(tk$^-$) cell line (thymidine kinase-deficient) with GES (L.GES) or LM (L.LM) cells were selected in DMEM plus HAT while non-adherent GES and LM cells were eliminated by washing. Hybrids obtained by the fusion of L(tk$^-$) and WISH cell lines (L.W) were selected on DMEM containing HAT plus 1.0 mg/ml G418 (Geneticin; GIBCO, Rockville, MD) since the adherent WISH cells were made G418-resistant by transfection with a pSV2neo plasmid (30) by calcium phosphate precipitation (31) prior to the fusion

PCR

All PCR reactions were performed on a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT) with the following cycles: one cycle of 4 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 55–65°C, 2 min at 72°C and finally one cycle of 10 min at 72°C. For each PCR reaction, the annealing temperature used was near to the melting temperature of each primers. Human genes were amplified with the following human-specific sense (S) and antisense (AS) primers sets: CIITA: H.C-S (5′-CGCCCTATTTGAGCTGTC-3′) and H.C-AS (5′-GGTC-AATGCTAGGTACTG-3′); RFX-5: HRFX5-S (5′-CCAATCCC-AGTCTCTCCAC-3′) and HRFX5-AS (5′-CACCACCTATGC-CTACCTCT-3′); RFXAP: HRFXAP-S (5′-GTTCTCATCAAGG-GTCAATT-3′) and HRFXAP-AS (5′-TAGTTAATCCGTCAG-GTTTT-3′); protamine I: PRM1-S (5′-GCTTGGCACACTAG-

Table 1. Sequence and position of the different primers used for the amplification and sequencing of the CIITA cDNA

Primer designation	Primer sequence	Position (nucleotides)
CM37S	5′-CTCAGAAGCACGGGGCACAG-3′	53–73*
CM10S	5′-GGAGACTTCCGGCACGCCAG-3′	24–43*
CM11S	5′-CGACCCCCTACATCTCTACC-3′	218–238
CM12AS	5′-CTGAGGCTGCTTGAAGGGAG-3′	758–738
CM43S	5′-CTCCCTTCAAGCAGCCTCAG-3′	739–758
CM2S	5′-TCAAGCAGCCTCAGTAT-3′	745–761
CM2AS	5′-TCTAGGATGAGCAGAAC-3′	1433–1417
CM15.1S	5′-GAGCGATTCCAGCACTCCCT-3′	952–971
CM32AS	5′-CTGCAGTCACTGACAACCTG-3′	1160–1140
CM13S	5′-GCCAGACCGTGTTCTGCTCA-3′	1407–1426
CM14AS	5′-CGGGAGGACTGTTCTGAGCT-3′	1900–1881
CM22S	5′-GAGGCTGCACACTGCTCCTCAC-3′	1553–1574
CM22AS	5′-GAGCCTCCGTGG TCTCCAGGGT-3′	2050–2028
CM23S	5′-CATCCGTGGAGGTGAAAACCTG-3′	1978–2000
CM23AS	5′-CTCCCTGCTGCTGAAGGGACTC-3′	2647–2626
CM24S	5′-GCTAGCCCACGGTGGTCTGGCA-3′	1110–1131
CM24AS	5′-CAGTAGCTTCCGCTGGAAGATC-3′	1551–1530
CM5S	5′-TGGCGTTGAGCCTTCTGGAC-3′	2535–2554
CM5AS	5′-CCGGCAGCCGTGAACTTGTT-3′	3200–3180
CM6S	5′-GGAGCCAAGAGCCTGGCACA-3′	3115–3134
CM6AS	5′-CCCTTAGCGTCTTCAGAGCC-3′	3786–3766
CM15S	5′-GGGCTGCCTTCCGTGGGTTC-3′	3607–3626
CM16AS	5′-CCCAGACAGCAACTTGTGTC-3′	4280–4260
CM27S	5′-ACACTCTGCATGCGCCCGTGTGC-3′	4450–4472
CM27AS	5′-CCCTGAGCCCAAGGTCCTAAC-3′	4989–4968
CM30S	5′-ATCTCTAGATGTGACAGGGAGCC-3′	3803–3825
CM30AS	5′-GCACACGGGCGCATGCAGAGTGT-3′	4472–4450

Numbering follows the sequence reported in (52) except for the primers noted with an asterisk, where the numbering is from (18).

GAGCTATTAG-3′) and PRM1-AS (5′-CCCTTGCCTTGCC-TGTAAAGCATG-3′); adenine phosphoribosyl transferase: APRT-S (5′-GGTGCATGTCACTGGCCTTTCAGCT-3′) and APRT-AS (5′-TGTCT CAACCTCTCTGAGCTCCCAA-3′).

RT-PCR

Total RNA was isolated from subconfluent cultures using the guanidinium thiocyanate method (32). One microgram of total RNA was incubated for 1 h at 42°C in the presence of 200 ng anti-sense oligonucleotides, 2 U AMV reverse transcriptase (Promega, Madison, WI), 15 U RNAsin, 1 mM dNTP and 2.5 mM $MgCl_2$, in the Taq buffer from the AmpliTaq kit (Perkin-Elmer Cetus), in 20 μl final volume. PCR reactions were performed on the resulting cDNAs in a 100 μl final volume (200 μM dNTP; 2.5 mM $MgCl_2$) after addition of the sense oligonucleotides and 5 U Taq DNA polymerase. PCR were performed on a DNA thermal cycler (Perkin-Elmer Cetus) using the amplification conditions: one cycle of 5 min at 95°C, 30 cycles of 1 min at 95°C, 1 min at 55–65°C, 2 min at 72°C and one cycle of 10 min at 72°C. For each PCR reaction, annealing temperature was close to the melting temperature of each primer set. Mouse *C2ta* mRNA was amplified using the sense and antisense primers described in Table 1. Amplification efficiency was assessed with non species-specific β-actin primers (15 ng) (β-Act-S: 5′-CACCCTGTGCTGCTCAC-CGAGGCC-3′; β-Act-AS: 5′-CCACACAGAGTACTTGCGC-TCAG G-3′) (17) added to each RT-PCR reaction.

Semiquantitative RT-PCR

Reverse transcription and PCR were performed as described above using the set of primers M3′-S (5′-CTGGATCGTCT-CGTGCAG-3′) and M3′-AS (5′-CCATGTCCGGAAGTACTT-3′) except that amplification was for 20 cycles and that 1 nCi/ml [^{32}P]dCTP was added per tube. The 424-bp amplification product (nucleotides 2728–3152 of the mouse CIITA cDNA) (33) was resolved on a 6% PAGE and autoradiographed.

RACE-PCR

RACE-PCR assays were performed with the 5′-RACE-PCR kit (Gibco/BRL) according to the manufacturer's instructions. CIITA cDNAs were obtained by RT-PCR using CM12-AS primer (5′-CTGAGGCTGCTTGAAGGGAG-3′, nucleotides 758–738). TdT-tailing of cDNA was carried out for 1 h at 4°C by incubating 10 μl of the resulting cDNA, 200 μM dCTP, 5 μl 5 × tailing buffer and 1 μl of TdT. A specificity control reaction was performed in the absence of TdT. For PCR amplification, CM20-AS primer 5′-TGCTGAACTGGTCGCAGTTGATGG-3′ (nucleotides 331–308) and Abridged Anchor Primer (AAP; Gibco) were used following the procedures described above. Nested-PCR was performed using 1/10 dilution of the PCR amplification product and CM21AS primer 5′-AGGTA-GCTGCCCTCTGGAGATCC-3′ (nucleotides 197–175) and Abridged Universal Amplification Primer (AUAP; Gibco).

Sequencing

Sequencing of each RT-PCR product was performed with a DNA sequencer 370A (Perkin-Elmer Cetus). Fluorescent deoxynucleotide labeling of DNA was performed by PCR with the ABI prism kit, following the manufacturer's instruction (Perkin-Elmer Cetus). The primers used to sequence each RT-PCR product were the same than those used for the RT-PCR experiments.

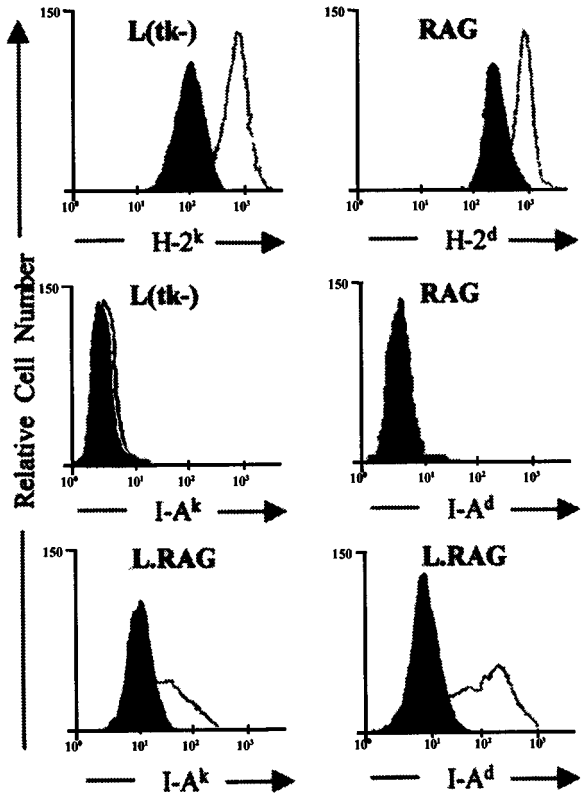

Fig. 1. Flow cytometric analysis of the RAG, L(tk⁻) and RAG × L(tk⁻) cell lines for the cell surface expression of MHC molecules. Cells were either untreated or treated with 250 U/ml of IFN-γ for 72 h. H-2^k and H-2^d molecule expression was detected with mAb 11.4.1 and 20.8.4 respectively. The MKD6 and H39.64.5 mAb were used to identify the I-A^d and I-A^k molecules respectively.

Immunoprecipitation and Western blot of CIITA

Immunoprecipitation was made with a polyclonal rabbit anti-human CIITA directed against a peptide (^{726}GEIKDKELPQYLALTR^{741}) described in (34) and immunoaffinity purified through a CNBr-activated Sepharose 4B column (Amersham Pharmacia Biotech, Piscataway, NJ) coupled to the peptide. Western blots were performed with a mAb anti-human CIITA clone 7-1H from R & D Systems (Minneapolis, MN). Cells were incubated with mouse IFN-γ (250 U/ml) and lysed either in RIPA buffer or in high salt buffer (10 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA and 5% glycerol) containing 0.5% NP-40 (Sigma). Lysates were cleared for 2 h with Protein G–Sepharose (Amersham Pharmacia Biotech) and CIITA proteins were next immunoprecipitated overnight with 2.5 μg of affinity-purified polyclonal anti-CIITA antibodies per sample. Immune complexes were recovered by binding for 30 min to Protein G–Sepharose, resolved on a 6% SDS–PAGE and absorbed to a nitrocellulose membrane (Hybond ECL; Amersham Pharmacia Biotech). Blocking was performed overnight in PBS/Tween buffer containing 2.5% low-fat milk. Incubation with 1.5 μg/ml of mAb anti-CIITA was performed for 2 h at room temperature in PBS/Tween buffer containing 0.25% low-fat milk. Immunoreactive bands were visualized with the ECL Plus Western blotting system (Amersham Pharmacia Biotech). To test for proteolytic activity in the RAG cell line, 2 mg of total cell extracts of RAW264.7 and RAG cells was mixed and incubated for 1 h at 37°C with or without proteases inhibitors from Sigma (0.4 mM PMSF, 3 μg/ml aprotinin, 1 μg/ml leupeptin and 0.5 μg/ml pepstatin A) followed by immunoprecipitation as described above. Moreover, the same type of experiments were performed using 10 μM of the proteasome inhibitor NLVS, kindly donated by Dr Hidde Ploegh (Boston, MA)

Northern blot analysis

Total RNA was isolated by the guanidinium thiocyanate procedure. Northern blot analysis was performed as previously described (35). The IFN regulatory factor (IRF)-1 and IRF-2 full-length cDNA probes were kindly provided by Hitoshi Ueda (Hyogo, Japan), and the guanylate binding protein (GBP)-1 cDNA probe was kindly provided by Mark Richter (Paris, France).

Luciferase constructs and assays

Plasmid constructs were made in the pGL3-basic vector from Promega (Madison, WI). The 445 bp pIV promoter fragment was obtained by PCR on mouse genomic DNA from L(tk⁻) and RAW264.7 cells using the primer set P1-S (5′-GGTTGG-GCTGAGATAGAGTG-3′) and P1-AS (5′-CTGTGCCCCGT-GCTTCTGAG-3′) designed after the published sequence (18). Both PCR fragments were subcloned in the *Sma*I site of pGL3-basic and sequenced. The resulting constructs were called PIV-L(tk⁻).1, PIV-L(tk⁻).8 and PIV-RAW5.1. Assays were performed with the luciferase assay system from Promega as described previously (19). The pGL3-control vector contains the SV40 promoter driving the luciferase expression. Transfection efficiency was monitored using the pSVβ-galactosidase vector from Promega.

Results

MHC class II-defective RAG and L(tk⁻) cell lines display different genetic defects

The RAG and L(tk⁻) cell lines do not express MHC class II genes after IFN-γ treatment even though IFN-γ induces MHC class I cell surface expression in both cell lines (Fig. 1). In order to determine if RAG and L(tk⁻) display defects in the same gene, RAG × L(tk⁻) hybrid cells were generated. These hybrids were analyzed by indirect immunofluorescence for surface MHC class II expression using two allele-specific mAb that allowed us to distinguish between MHC class II molecules from each cell lines [haplotypes d and k for RAG and L(tk⁻) respectively]. RAG × L(tk⁻) hybrids display cell surface expression of both I-A^d and I-A^k MHC class II molecules when treated with IFN-γ demonstrating that *I-A* genes are functional in both cell lines (Fig. 1). Additionally, we conclude from these results that the RAG and L(tk⁻) cell lines display mutations in different genes, and that both defects are recessive.

Table 2. Differences in nucleotide sequences obtained for the RAW264.7, RAG and L(tk⁻) cell lines as compared with the published sequence (33)

RAW264.7		RAG		L(tk⁻)	
Nucleotide position	Amino acid position	Nucleotide position	Amino acid position	Nucleotide position	Amino acid position
G454C	E121Q	G454C	E121Q	G1071T	Q326H
A458T	H122L	–	–	C1549T	silent
T795C	silent	T795C	silent	C1983A	silent
C1045T	silent	–	–	G2166T	K691N
G1171A	E363K	–	–	G2735A	R881H
		G4405A	3′UTR		
		G4414C	3′UTR		

The putative changes in amino acid residues are also indicated. The numbering of nucleotides and amino acids follows that published in (33). 3′UTR = 3′ untranslated region.

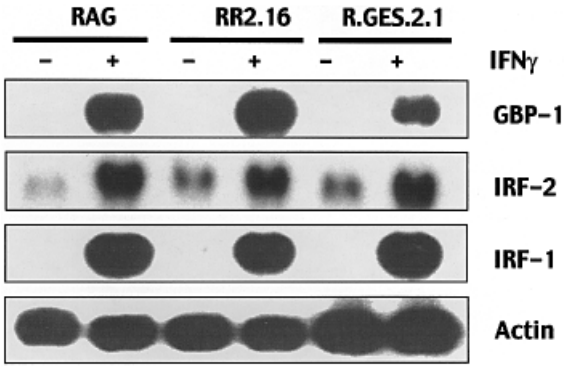

Fig. 2. Northern blot analysis for the expression of several IFN-γ-responsive genes, *GBP-1*, *IRF-1* and *IRF-2*, in the RAG cell line. Total RNA was isolated from cells either untreated or treated with 250 U/ml of IFN-γ for 24 h. Hybrids R.GES2.1 and RR2.16 were used as positive controls.

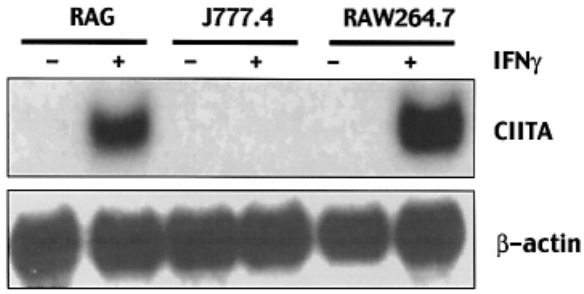

Fig. 3. Semiquantitative RT-PCR analysis of *C2ta* expression in the RAG cell line. Total RNA was isolated from cells either untreated or treated with 250 U/ml of IFN-γ for 6 h. The monocyte/macrophage RAW264.7 cell line was used as a positive control. The J777.4 pre-monocyte cell line was used as a negative control. [^{32}P]dCTP was introduced in the reaction mix and a 20-cycle amplification was applied. Amplification products were run on a 6% acrylamide gel and autoradiographed. Mouse β-actin primers were introduced in the reaction to control the amount of cDNA per lane.

The RAG cell line

Expression of C2ta transcripts in the RAG cell line. We have previously described that MHC class II expression can be restored in RAG cells by transfection of the human CIITA cDNA. However, analysis of hybrid cells between RAG and CIITA-deficient cell lines indicated that *C2ta* was not mutated in the RAG cell line (26). These data therefore suggest that a factor required for CIITA expression might be missing or defective in these cells.

Defects in *MHC2TA* transcription associated with a general alteration on the IFN-γ transduction pathway have been described for different tumor cells. Indeed, defects on the transcripts of *GBP-1* and *MHC2TA* were found after IFN-γ induction of the non-small cell lung cancer and small cell lung cancer tumor cell lines (23). We therefore investigated if the IFN-γ transduction pathway was fully functional in the RAG cell line through the analysis of different IFN-responsive gene expression. The hybrid cell lines R.GES.2.1 and RR2.16, which express MHC class II molecules after an IFN-γ incubation, were used as positive controls. These somatic cell hybrids were previously described and they were generated by the fusion of RAG cells with two different human cell lines. They contain human chromosome 16 or fragments of this chromosome (R.GES2.1 and RR2.16 respectively) and we have demonstrated that the recovery of mouse MHC class II expression was correlated with the presence of this chromosome (25). The expression of the *IRF-1*, *IRF-2* and *GBP-1* IFN-γ-responsive genes was assayed through Northern blot analysis. Figure 2 demonstrates that all three genes are inducible in RAG cells with similar intensities when compared to the somatic cell hybrids. Therefore, the defect in the RAG cell line does not affect the IFN-γ transduction pathway and is limited to the expression of MHC class II molecules.

We next examined the transcription of *C2ta* in RAG through a semiquantitative RT-PCR assay (20 cycles). As controls we used RNA from mouse CIITA⁺ (RAW264.7) and CIITA⁻ (J777.4) cell lines. As shown in Fig. 3, RAG cells display a significant amount of *C2ta* transcripts after induction with IFN-γ. These levels of *C2ta* mRNA are only slightly weaker than

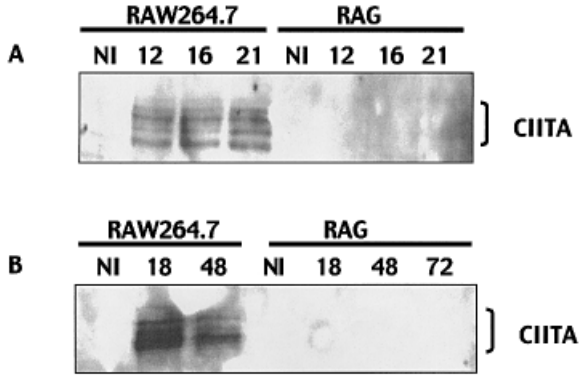

Fig. 4. Expression of CIITA protein is not observed in RAG cells. Expression of the CIITA protein was studied by immunoprecipitation with a purified polyclonal rabbit anti-CIITA antibody and revealed by Western blot using a mAb directed against CIITA. RAG or RAW264.7 cells were untreated or treated with 250 U/ml of IFN-γ for different times. (A) Cells were lysed in a high salt buffer. (B) Cells were lysed in RIPA buffer containing SDS. Immunoprecipitations were performed using 660 μg of extracts. Data are representative of three independent experiments.

those detected in RAW264.7, as expected from the monocytic origin of the latter. In addition, the lack of *C2ta* transcript in the J777.4 pre-monocytic cells demonstrates that the CIITA fragment amplified in RAG and RAW264.7 cells is specific.

In order to assess if in RAG cells the *C2ta* messenger results from an appropriate transcription initiation site, 5′-RACE-PCR was performed. The RAW264.7 and the B lymphocyte A20 cell lines were used as positive controls for transcription initiations on promoters IV and III respectively (18,19). Nucleotide sequencing identified the amplification products obtained from three independent experiments. The amplification products obtained from the A20 cell line were found to correspond to transcripts initiated from promoter III at position –1. In IFN-γ-treated RAW264.7 cells, transcription was initiated from promoter IV at positions –13, –14 or –15. In the RAG cell line, transcription was initiated from promoter IV at positions +16 or +32. Since initiation of *C2ta* transcription from promoter IV can take place within positions –54/+69 with two major initiation sites at positions +1 and +17 (18), we conclude that transcription of *C2ta* in RAG is initiated from promoter IV using adequate start sites for its expression.

Abnormal splicing events within *C2ta* transcripts in RAG might account for its inability to induce the expression of MHC class II molecules. We thus amplified through RT-PCR the CIITA cDNA from RAG cells using 13 sets of primers allowing the recovery of short and overlapping amplification products. For each set of primers we found that the size of the products was similar in RAG and in RAW264.7 cells, thereby suggesting the absence of major abnormalities in the splicing of *C2ta* (data not shown). In order to assess the absence of minor deletions that could take place during the splicing in the *C2ta* mRNA, all PCR amplification products from both cell lines were directly sequenced without further subcloning. This approach has the additional advantage to permit the detection of putative heterozygous mutation. Differences found in *C2ta* sequences of both the RAW264.7 and RAG cDNAs compared to the published sequences are summarized in Table 2.

All together, our data converge towards the demonstration that the transcription of *C2ta* in the RAG cell line is comparable to that observed in a monocytic cell line in which the expression of MHC class II is normally induced by IFN-γ. This suggested that the defect in MHC class II expression in RAG cells is not the result of a defect in *C2ta* transcript, leading us towards the hypothesis of a defect in the biosynthesis or stability of CIITA protein in this cell line.

Absence of the CIITA protein in the RAG cell line. The expression of CIITA was analyzed through immunoprecipitation and detection by Western blot with a mAb directed against the protein. As shown in Fig. 4(A), multiple CIITA forms were detected in the control RAW264.7 cell line treated with IFN-γ for 12–21 h, as previously observed in human cell lines (Barbieri *et al*., submitted and our unpublished data). However, CIITA was not detected in cell extracts from RAG cells. Following the hypothesis that CIITA might be improperly folded or located in a specific compartment in the RAG cells that would hamper its solubilization in mild detergents, the immunoprecipitation was also performed using RIPA lysis buffer that includes SDS (Fig. 4B). Here again CIITA could not be detected in RAG cell extracts, even when increasing the IFN-γ induction period up to 72 h. These data thereby demonstrate that even though *C2ta* transcripts are present, the CIITA protein is not detectable in the RAG cell line.

A high abnormal proteolytic activity might account for the lack of CIITA protein in RAG cells. To test this point, we analyzed the stability of the CIITA protein expressed by the RAW264.7 cells in the presence of different amounts of cytosolic extracts from RAG cells prepared using mild detergent conditions known to preserve proteolytic activity (36). These experiments did not reveal any evidence of an enhanced degradation of the CIITA protein by RAG cell extracts (data not shown).

The L(tk⁻) cell line

Complementation of MHC class II expression. We have already shown that the mouse fibrosarcoma cell line L(tk⁻) does not express MHC class II molecules after IFN-γ treatment (37). Moreover, transfection of *MHC2TA* genomic DNA in the L(tk⁻) cells was shown to restore cell surface I-A expression (17), thereby suggesting that L(tk⁻) cells are defective in *MHC2TA*. This led us to study further the defect in MHC class II expression in the L(tk⁻) cell line.

We prepared several somatic cell hybrids between L(tk⁻) cells and human B lymphoblastoid cell lines (GES and LM) or with the human amniotic cell line WISH. Out of the 13 hybrid clones and subclones analyzed here, eight were induced by IFN-γ to express mouse MHC class II molecules (Fig. 5 and data not shown). Consequently, human genetic material is able to complement the expression of MHC class II in L(tk⁻) cells.

It has already been shown that in human × mouse hybrids, human chromosomes are segregated over the mouse genetic background (38). This phenomenon can be used to identify the human chromosomes which allow the restoration of a normal phenotype in mutated mouse cell lines (25,38). However, this methodology could not be applied here as the

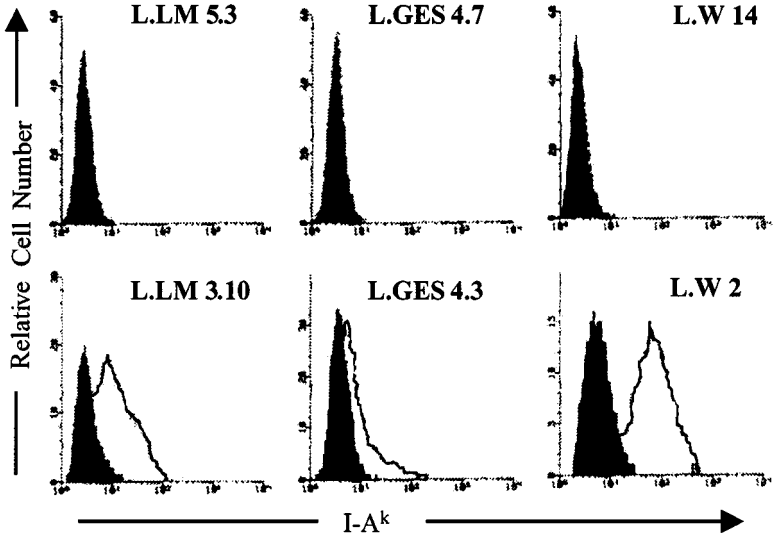

Fig. 5. Flow cytometric analysis of I-A^k cell surface expression in somatic cell hybrids (L.GES, L.W or L.LM) obtained by the fusion of the L(tk$^-$) cells with different human cell lines (GES, WISH or LM). Cells treated with 250 U/ml of IFN-γ for 72 h are represented as light histograms. Untreated cells appear as dark histograms.

Table 3. Correlation between the inducible expression of MHC class II molecules in a panel of L(tk$^-$) somatic cell hybrids and the presence of human genes implicated in MHC class II regulation

Cell hybrids	Mu-MHC II induction	Hu-CIITA	Hu-PRMI	Hu-APRT	Hu-RFX-5	Hu-RFX-AP
L.LM3.30	+	+	+	+	+	+
L.LM3.10	+	+	+	–	+	+
L.GES 4.3	+	+	+	+	–	+
L.W 2	+	+	+	+	+	+
L.W 7B-3	+	+	+	–	–	–
L.W 6	+	+	+	+	+	+
L.W 10.1 B-1	+	+	+	–	–	–
L.W 1	+	+	+	+	+	+
L.LM 5.3	–	–	–	–	–	–
L.GES 4.7	–	–	–	–	+	+
L.W 14	–	–	–	–	+	–
L.W 10.1C-2	–	–	–	–	–	–
L.W 7.2 C-4	–	–	–	–	–	+

hybrids with the L(tk$^-$) background contained multiple fragmented human chromosomes, thereby hampering any significant analysis through karyotyping. Therefore, we analyzed by PCR the segregation of human *MHC2TA* in the whole panel of somatic cells hybrids described above. Two other genes, encoding protamine I (*PRMI*) (16p13.13) and adenine phosphoribosyl transferase (*APRT*) (16q24.2), located on human chromosome 16 were used as markers for this chromosome. Results are shown in Table 3. A positive correlation was evidenced between the presence of human *MHC2TA* and *PRMI* and expression of mouse MHC class II molecules. No association was found with other transcription factor encoding genes (*RFX5* and *RFXAP*) described to be mutated in MHC class II-defective cells nor with the *APRT* gene. These results are in agreement with a mutation in the *C2ta* gene in the L(tk$^-$) cell line.

Analysis of CIITA expression in the L(tk$^-$) cell line. The analysis of *C2ta* transcription in the L(tk$^-$) cell line revealed a high degree of complexity. The level of *C2ta* transcripts was much lower in the L(tk$^-$) cell line than that observed in the RAW264.7 or in the RAG cells (Fig. 6). Indeed, when examining the activity of *C2ta* promoter IV through luciferase reporter gene experiments, the L(tk$^-$) cells were 4 times less active than the RAW264.7 cells when IFN-γ was used to induce transcription from this promoter (Fig. 7). In contrast, the activity of the SV40 promoter did not present any major difference between both cell lines (Fig. 7, pGL3-control vector).

In addition, depending on the primer set used, the relative level of expression of *C2ta* transcripts in L(tk$^-$) compared to the RAW264.7 cells was highly variable (Fig. 6). This phenomenon was particularly visible in the 5′ region of the CIITA

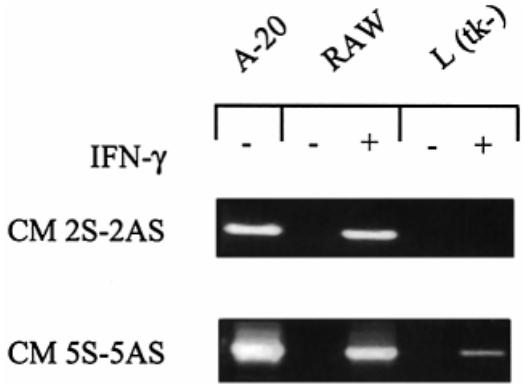

Fig. 6. RT-PCR analysis of *C2ta* mRNA expression in the L(tk⁻) cell line. RAW264.7 and A20 cells were used as positive controls. Total RNA was isolated from cells either untreated or treated with 250 U/ml of IFN-γ for 6 h. Primers CM2S-2AS and CM5S-5AS allow the amplification of products 741–761 and 1433–1417 nucleotides respectively.

cDNA in L(tk⁻) where amplification of the cDNA was not observed with primer sets hybridizing on the first 950 bp of the *C2ta* transcript. RACE-PCR did not allow the precise determination of an intragenic transcription initiation site. A highly G-rich region and the weak amount of transcripts hampered this methodology. We additionally did not evidence alternative splicing events with transcripts initiated from either promoter III and IV. The sequencing of both alleles of promoter IV of *C2ta* did not reveal any mutation. Nucleotide sequencing of the CIITA cDNA from L(tk⁻) cells (nucleotides 952–4989) revealed different substitutions in the coding region compared to the published sequence which are summarized in Table 2. Moreover, a deletion of two out of 14 repetitive motifs (ccaaaccaaa) present in the non-coding cDNA sequence was found. Immunoprecipitation of the CIITA protein was next attempted using L(tk⁻) cell extracts. As seen in Fig. 8, and in agreement with the above observations, the protein could not be evidenced in the L(tk⁻) cell extracts whatever induction period with IFN-γ (16–48 h) was applied.

Taken as a whole our data indicate that the L(tk⁻) cell line presents altered *C2ta* transcripts and is devoid of the CIITA protein.

Discussion

Loss of MHC class II expression has been observed in numerous types of tumor cell lines: small cell lung cancers (39), non-small cell lung cancers, neuroblastoma (40), cervical carcinoma and retinoblastoma cell lines (23), pancreatic tumors (41), certain melanoma cell lines (24), choriocarcinoma (42) or multiple myeloma (43). In the latter two cases MHC class II expression has been shown to be repressed in association with the differentiation state of the cell lines (42,43). In contrast, the other tumor cells were expected to express MHC class II following IFN-γ stimulation. Several of them (neuroblastoma, retinoblastoma or plasmacytoma cell lines, small cell lung cancers and pancreatic tumors) were shown to lack *MHC2TA* transcription (23,39,40,41). However the defects causing the lack of *MHC2TA* transcription have not been established, except for the pancreatic tumor cells and a corresponding cell line, where a mutation in the *IRF-2* gene has been evidenced (41). Therefore, we have studied two mouse cell lines presenting a defect in MHC class II expression in order to define more precisely the types of mutations that can affect tumor cells.

Different from the above cell lines, the L(tk⁻) fibrosarcoma cell line displays a weak amount of *C2ta* transcripts. Interestingly, defects hampering CIITA expression have been observed at different levels in this cell line. We had previously shown that the L(tk⁻) cells exhibit a poor induction by IFN-γ of certain IFN-γ-responsive genes [genes 1–8 and (2′–5′)oligoadenylate synthetase] (37), although it is normally responsive for the stimulation of MHC class I molecules (17,37). However, the IFN-α response is unaltered (37), suggesting that this cell line displays a selective and partial defect in the IFN-γ transduction pathway. In agreement with this phenotype, the expression of *C2ta* promoter IV driving a luciferase reporter gene was reduced 4-fold in the L(tk⁻) cells when compared with the RAW264.7 cell line (Fig. 7). Defects in the IFN-γ transduction pathway is not a rare phenomenon in tumor cell lines, although to our knowledge these defects usually totally inhibit the IFN-γ response, as shown in cervical carcinoma cell lines (23), pancreatic tumors (41), melanoma (44) or renal cell carcinoma cell lines (45).

In addition to the poor *C2ta* transcript expression in the L(tk⁻) cell line, we have shown that *C2ta* transcripts are truncated of ~950 nucleotides in their 5′ region. RACE-PCR techniques have not allowed the identification of the actual transcription initiation site of these transcripts. They might either originate from illegitimate transcription start site(s) or from an intragenic promoter as suggested in a previous report (19). Based on these data we have attempted to identify a truncated form of the CIITA protein which was not detected (data not shown).

Our data show that transcription from promoter IV of *C2ta* does not occur in a detectable fashion in the L(tk⁻) cell line. Data from this report and (17) suggest that the defect of the L(tk⁻) cell line resides in the *C2ta* gene, thus we examine the existence of mutations in the L(tk⁻) *C2ta* promoter IV. We show here that a 445-bp promoter fragment from L(tk⁻) drives normal expression of the luciferase reporter gene in the RAW264.7 cell line (Fig. 7) and displays a normal nucleotide sequence (data not shown). The lack of expression from promoter IV might then be explained by mutations outside the minimal promoter. However, it is possible that the *C2ta* endogenous promoter is not functional due to an epigenetic phenomena. Indeed, in a human choriocarcinoma cell line, methylation of *MHC2TA* promoter IV has been shown to inhibit *MHC2TA* transcription and thereby the expression of *HLA-D* genes (42). Alternatively, binding of histone deacetylases to *MHC2TA* promoter IV might have similar effects on CIITA expression, as exemplified in a plasmacytoma cell line (40). Blockade of *C2ta* promoter IV might then favor a residual transcript initiation from an intragenic position or illegitimate transcription initiation(s). In summary, in addition to different mutations (or polymorphisms) in the CIITA cDNA sequence, alterations have been observed in the L(tk⁻) cell line with a lack of transcription from the promoter IV and partial modifications in the response to IFN-γ.

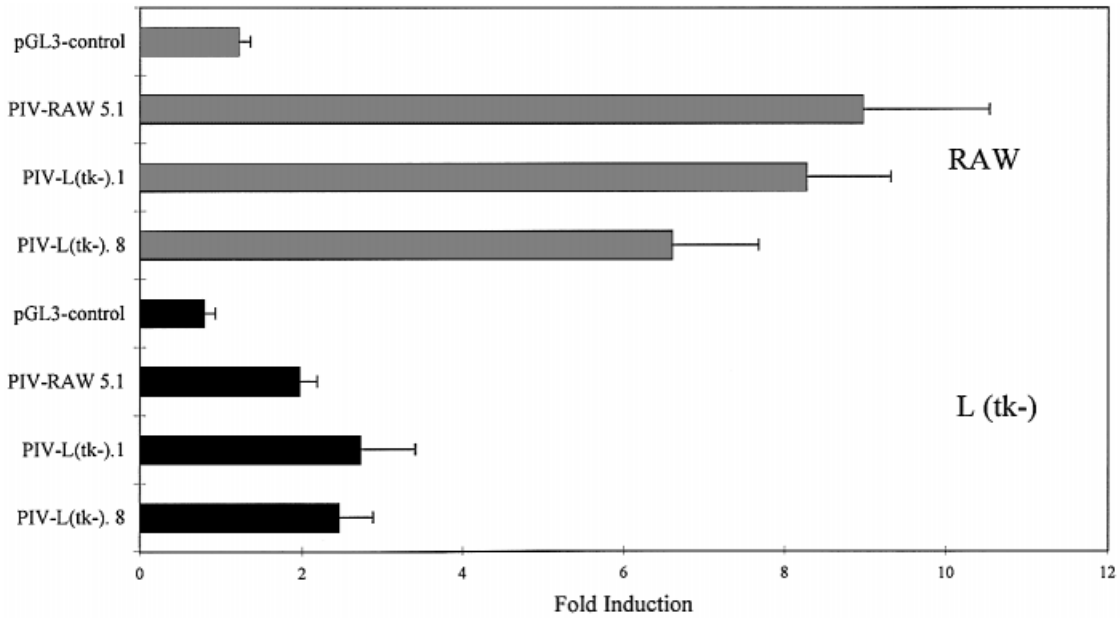

Fig. 7. IFN-γ-induced *C2ta* promoter activity. The IFN-γ-induced *C2ta* promoter (PIV) from RAW264.7 cells (PIV-RAW 5.1) and L(tk⁻) cells [PIV-L(tk⁻).1 and PIV-L(tk⁻). 8]. were cloned in a luciferase reporter vector and co-transfected with pSVβ-galactosidase plasmid in RAW264.7 and L(tk⁻) cell lines. After a 24 h of induction with 100 U/ml of IFN-γ, cell extracts were assayed for luciferase and β-galactosidase activity. Luciferase intensity values were standardized for transfection efficiency with the co-transfected pSVβ-galactosidase plasmid. Values are expressed as fold induction by dividing the luciferase activity value from IFN-γ-treated cells by the luciferase activity from untreated cells. IFN-γ-response specificity was assessed with the pGL3-control vector, in which the SV40 promoter controls the luciferase gene. Results are the average of three independent experiments.

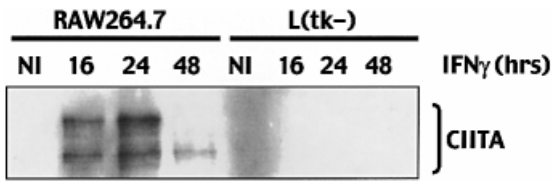

Fig. 8. Expression of CIITA protein is not observed in the L(tk⁻) cells. Expression of the CIITA protein was studied by immunoprecipitation with a purified polyclonal rabbit anti-CIITA antibody and revealed by Western blot using a mAb directed against CIITA. Cells were untreated or treated with 250 U/ml of IFN-γ for different times. Immunoprecipitations were performed using 660 μg of extracts. Data are representative of three independent experiments.

In contrast, RAG cells have retained the transcription of the *C2ta* gene after treatment with IFN-γ, even though they lack MHC class II expression. Interestingly, this unusual phenotype has been described in another mouse adenocarcinoma cell line (40). Our data assess that the RAG cell line is not defective for the *C2ta* gene and that the IFN-γ transduction pathway is functional. In addition, the cDNA CIITA nucleotide sequencing has confirmed the absence of mutation in the coding region and the lack of splicing defects. RACE-PCR experiments have demonstrated that transcription initiation occurs within sites that have been described previously in normal cell lines. All together our data show that the *C2ta* transcript of the RAG cell line is expressed and that it is devoid of defects. However, we have not shown that the factors required, downstream of CIITA, for MHC class II gene expression were functional in this cell line (35).

We have shown in this report that the CIITA protein is not detected in the RAG tumor cell line, in agreement with the lack of MHC class II transcription and cell surface expression. Different hypotheses are compatible with our data. One possibility is that the *C2ta* transcript is not translated either through a blockade of its nuclear export or through the lack of a factor required for the translation of CIITA. It is also possible that the CIITA protein is expressed; however, in such a labile manner that we did not evidence this protein. Data obtained on cells infected with *Chlamydia* showed that this organism expresses a protease degrading RFX5 and up-stream stimulatory factor (USF)-1 (36,46), and thereby inhibits MHC class II expression in the infected cells. As the RAG cell line could be infected by an oncogenic virus presenting similar capacities, a highly active viral protease might degrade the CIITA protein. This phenomenon was not evidenced in this report, in agreement with the recessivity of the RAG mutation that does not fit with this hypothesis. We also hypothesized that CIITA might be rapidly degraded by an over-active immune proteasome in the RAG cell line. However, treatment of these cells with a proteasome inhibitor did not alter MHC class II expression, although it reduced MHC class I expression (data not shown). This suggests that *C2ta* and MHC class II gene-specific transcription factors are not submitted to proteasome degradation. One additional possibility is that a specific chaperone, allowing the proper intracellular trafficking or editing of the CIITA protein, is inactive or missing in the RAG cells. Interestingly, we have shown that the transfection of a

human CIITA cDNA allows for the immunoprecipitation of the human CIITA protein (data not shown) and restores MHC class II expression. This might indicate that the over-expression of a recombinant form of CIITA bypasses these putative defects or that the missing or mutated factor is not required for the human form of the CIITA protein or transcript. Even though we have shown that both the *C2ta* gene and transcript are normal in the RAG cell line, numerous hypotheses remain concerning the defect on these cells, as this defect can affect various stages of CIITA protein expression as proposed above. Further study of this cell line will therefore help in the understanding of CIITA synthesis and MHC class II expression.

The importance of MHC class II molecule expression in tumor cells is quite controversial (21,47,48). The loss of MHC class II molecules is not essential with regard to immune escape. Recent data suggest that the lack of CIITA itself might be the crucial step. Indeed CIITA, through the sequestration of NFAT (49,50), has been shown to modulate the expression of genes like Fas ligand (49) or IL-4 (50). In addition, CIITA can interact with cyclins (51) or the transactivator CBP (11). One can therefore hypothesize that CIITA, through an indirect effect, might alter tumor growth, tumor progression or immune escape. The loss of CIITA expression might therefore confer a selective advantage to tumors from certain cell types. This thereby reinforces the necessity of the identification of the defects altering CIITA expression in tumor cell lines and the precise targets of CIITA effects.

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Abbreviations

APC	antigen-presenting cell
CBP	CREB binding protein
CIITA	class II transactivator
CREB	cAMP-responsive element binding protein
GBP	guanylate binding protein
IRF	IFN regulatory factor
NFY	nuclear factor Y
RFX	regulatory factor X
USF	up-stream stimulatory factor

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