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Constitutive Expression of MHC Class II Genes in Melanoma Cell Lines Results from the Transcription of Class II Transactivator Abnormally Initiated from Its B Cell-Specific Promoter

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In melanoma cell lines, two different patterns of MHC class II expression have been described, either an IFN-γ-inducible expression of HLA-DR and HLA-DP, with a faint or null expression of HLA-DQ, resembling that described for melanocytes, or a constitutive expression, i.e., IFN-γ independent, of all three HLA-D isotypes. As this latter phenotype has been associated with a more rapid progression of melanoma tumors, we have analyzed in different melanoma cell lines the molecular mechanisms leading to this abnormal pattern of MHC class II expression. In agreement with the evidence of a coordinate transcription of the HLA-D genes in these cell lines, we have shown the constitutive expression of CIITA (class II transactivator) transcripts, CIITA being known as the master switch of MHC class II expression. Unexpectedly, these transcripts initiate from promoter III of the CIITA gene, a promoter that is mainly used constitutively in B lymphocytes. This expression was further shown to occur through factor(s) acting on the enhancer located upstream of CIITA promoter III, which was previously described in epithelioid cells as an IFN-γ-response sequence. The hypothesis of a general abnormality of the IFN-γ transduction pathway was dismissed. Constitutive transcription of CIITA from promoter III having been observed in unrelated melanoma cell lines, we propose the hypothesis that this phenomenon might not be a random event, but could be linked to the neoplastic state of the melanoma cells. The Journal of Immunology, 2001, 167: 98–106.

Melanoma arise from melanocytes that are pigment-producing cells derived from the neural crest, and represent the most lethal type of cutaneous cancers. As chemotherapy is poorly efficient on this type of cancers (1), mainly on distant metastasis, immunotherapy has been considered. With the evidence of specific tumor Ags presented by MHC class I molecules (reviewed in Ref. 2), vaccination of patients with tumoral peptides (3, 4) was undertaken. This was demonstrated to be efficient, even though moderated by results showing that these treatments might induce immune escape of the tumors through MHC class I loss of expression (5) or mutations in the vaccinated tumor Ag (4).

More recently, attention has focused on the role of CD4+ lymphocytes in tumor elimination (see Ref. 6 for a review) with the evidence of tumor Ag-specific CD4+ lymphocytes (7, 8). However, even though both CD4+ and CD8+ cells infiltrate the melanoma tumors (9), these cancer cells often acquire the capacity to escape immune surveillance through lymphocyte anergy via mechanisms that are not yet unraveled (10). In addition, melanoma cells often display a constitutive expression of MHC class II molecules (11, 12), which is usually restricted to professional APC (13). Although MHC class II presentation of tumor Ags by melanoma cells has been described (14), MHC class II expression was shown to be associated with a better progression of primary melanoma and a higher metastatic dissemination (15). MHC class II constitutive expression in melanomas is considered nowadays as a progression marker (16, 17). Therefore, it was proposed that MHC class II-expressing tumors might mimic an APC and induce lymphocyte anergy by the lack of accessory signals (18). Within the hypothesis of a role in the immune escape and tolerance induction, mechanisms leading to the constitutive expression of MHC class II Ags in melanoma cells must be investigated.

MHC class II molecules are represented by three isotypes, HLA-DR, HLA-DQ, and HLA-DP, consisting of a heterodimer of transmembrane glycoprotein α and β encoded by distinct genes. As mentioned above, constitutive expression of MHC class II molecules is restricted to professional APC such as dendritic cells, macrophages, and B lymphocytes. However, expression of MHC class II molecules is inducible by various cytokines, of which the most potent is IFN-γ in many of cells such as fibroblasts, epithelial cells (see Ref. 13 for a review), or melanocytes (19). In addition to a tissue-specific expression, MHC class II molecules are subject to a differential expression depending on cell activation with T lymphocytes or differentiation in...
plasmocytes (13). Defects in MHC class II molecule expression are responsible for a severe immunodeficiency syndrome (20), while aberrant expression of MHC class II in inappropriate tissues can lead to autoimmune diseases (21). Therefore, expression of MHC class II molecules requires a tight control of regulation.

The genes coding the MHC class II isotypes, in addition to the molecules involved in Ag presentation such as HLA-DM or the invariant chain (Ii), are usually coordinately regulated at the transcriptional level (reviewed in Ref. 22). Their promoters contain conserved motifs, the W, X1, X2, and Y boxes. The X1 box binds regulatory factor binding to X box (RFX), a heterotrimer composed of RFXANK (23), RFX-associated protein (24), and RFX5 (25). The X2 and Y boxes are binding sites, respectively, for a homodimer identified as CREB (26), and the nuclear factor binding to Y box heterotrimer (27). However, these ubiquitously expressed factors are not sufficient for the transcriptional activation of the MHC class II genes and their proper tissue-specific expression. The class II transactivator (CIITA) (28) is a non-DNA-binding protein. Through its interaction with RFX5, RFXANK, CREB, two subunits of the nuclear factor binding to Y box complex, and the RNA polymerase transcription machinery, CIITA creates the scaffold required for a correct conformation of the MHC class II gene promoters and their transcriptional activation (29). In addition, CIITA confers the tissue-specific expression of MHC class II molecules, with a constitutive expression in B lymphocytes (28) and dendritic cells, and an IFN-γ-responsive transcription in other cell lines (30). In addition to the tissue-specific expression of CIITA, it was demonstrated that differentiation can regulate CIITA expression, with the silencing of CIITA transcription in plasmocytes (31), and that different cytokines can regulate its expression (22).

The role of CIITA as a master switch of MHC class II genes has led to the analysis of its transcription. The CIITA gene is controlled by four different promoters located within a 13-kb DNA region (32). Promoter II has been described only in human cells, even though its function remains yet unknown. Promoters I and III drive, respectively, the constitutive expression of CIITA in dendritic cells and in B lymphocytes (32–34), while the promoter IV principally controls the IFN-γ-stimulated transcription of CIITA. These promoters direct the transcription of four different first exons connected to a common second exon. Both the second common exon and the first alternative exons expressed from promoters I and III contain putative translation initiation codons (32). Even though the second common exon contains a translation initiation codon, the CIITA proteins transcribed from promoters I and III might use upstream ATG located in the alternative first exons. The IFN-γ response of the CIITA gene is mediated by the phosphorylation of STAT1, which promotes the transcription of IFN regulatory factor-1 (IRF-1) and the binding of both IRF-1 and STAT-1 in association with USF-1 on proximal promoter IV (35, 36). Transcripts can also initiate in IFN-responsive cells from promoter III following a treatment with the cytokine. This phenomenon occurs through the presence of a 1068-bp enhancer element located ~5 kb upstream promoter III (34), to which we will refer as the 1-kb enhancer element. Through this 1-kb enhancer element, the response to IFN-γ induction is STAT-1 dependent, but IRF-1 independent (36).

The aim of our study is to understand the molecular mechanisms leading to the constitutive expression of MHC class II genes in melanoma. We will show in this work that this abnormal pattern of expression is due to the constitutive transcription of CIITA from promoter III, and is caused by trans-acting factor(s) acting on the 1-kb enhancer element upstream this promoter.

Materials and Methods

Cell culture

The HT144 (ATCC: HTB-63), 429/5, M74, and A375 (ATCC: CRL1619) human melanoma cell lines were kindly provided by E. Tartour (Institut National de la Sante et de la Recherche Médicale (INSERM) Unité 255, Curie Institute, Paris, France). BUA, an SV40-established human fibroblast cell line, and COM, an EBV-established human B lymphocyte cell line, were used as controls for the IFN-γ-inducible or constitutive expression of CIITA and MHC class II molecules, respectively, and were kindly provided by B. Lisowski-Grospiere (INSERM Unité 429-Hôpital Necker). All these cell lines were grown in RPMI 1640 supplemented with 10% heat-inactivated FCS, antibiotics, and 2 mM l-glutamine.

Flow cytometric analysis

Indirect immunofluorescence assays were performed with a FACScan (BD Biosciences, Mountain View, CA) using a CellQuest program on cells treated for 72 h with 250 nm IFN-γ (PeproTech, Rocky Hill, NJ). Before fixation of the primary antibody, the melanoma and BUA cell lines were preincubated with pure heat-inactivated FCS to block mAb-unspecific binding. The primary Abs were L243 (37), B7/21 (38), and L2 (39), respectively, directed against membrane HLA-DR, DP, and DQ Ags. Human melanoma MHC class I Ags were detected with the W6/32 Ab (Serotec, Oxford, U.K.). Cell labeling was then performed with the anti-mouse Ig labeled with FITC from Biosys (Compiègne, France).

Cell transfections, luciferase constructs, and assays

Melanoma cell lines were plated at a density of 4 × 105 cells/well in six-well plates. Twenty-four hours later, semiconfluent cells were cotransfected with a 5:1 molar ratio of a luciferase construct and pON1 plasmid. pON1 encodes the β-galactosidase gene placed under the control of the Rous sarcoma virus promoter (40), and allows the monitoring of transfection efficiency. The transfection of the melanoma cell lines was performed with the Effecten Reagent (Qiagen, Chatsworth, CA) following the instructions of the manufacturer. Twenty hours later, transfected cells were treated with IFN-γ for 24 h. Luciferase and β-galactosidase activities were assessed using the Luciferase Assay System and the β-galactosidase Enzyme Assay System kits from Promega (Madison, WI), according to the manufacturer’s instructions. Luciferase activity was measured with a BioOrbit luminometer (Lumat, Wallac), using a positive light unit per second (RLUs) 1 were further divided by the β-galactosidase activities (expressed as A420) to correct for transfection efficiency. The pGL3-control, containing an SV40 promoter upstream of the luciferase gene, and the promoterless pGL3-basic and pGL2-basic constructs were from Promega. The pGL3-DRA, pGL3-DQA, and pGL3-DFA plasmids contain 250-bp promoter fragments of the HLA-D genes, which include the conserved W, X1, X2, and Y boxes (41). The human CIITA-ApD2 vector (42) containing the minimal promoter IV of CIITA cloned in the pGL2-basic vector was kindly provided by E. Benveniste (University of Alabama, Birmingham, AL). The CIITA-1783-luc construct contains 1783 bp from promoter III of CIITA cloned in the pGL3-basic vector (33). The pLucCIITA Luciferase; pHIRED2CIITA Luciferase; pHIRED3CIITA Luciferase; and pHIRED4CIITA Luciferase constructs were described previously (34, 36) (see Fig. 9 as well). The pGL2-Del2 construct corresponds to 250 bp upstream of the CIITA Luciferase plasmid mutantized to replace the consensus Brn-2/N-Oct3 binding site (CATGCAGAATTG) located in the enhancer with the CATTG CAGCATG sequence demonstrated to inactivate Brn-2 binding (43). The mutantization was performed, following the instructions of the manufacturer with the Quick Change site-directed mutagenesis kit from Stratagene (La Jolla, CA), using the following primer (5′-TCTAGACACCTACCCCTCCATGCAGCATGCAGCTGTTGACGCT-3′). A deletion in the same site removing the AAAAT half of the palindrome was created in the pHIRED2-Brn-Del-Luc construct, following the same procedure with the primer 5′-CCCTAATCTTTACAACCTTACCCATGCTGTTGACGGTTGACT-3′.

The pGL3-SV40.Enh construct was generated through the ligation of the SacI-linearized pGL3-control vector (Promega) and a 2132-bp PCR product containing the enhancer upstream promoter III of the CIITA gene. The construct was cotransfected in the HT144 cell line, using Effecten as described above, with a Renilla luciferase reporter construct (pRL-TK vector from Promega) to control for transfection efficiency (molar ratio 1:5

Abbreviations used in this paper: Ii, invariant chain; CIITA, class II transactivator; CSPD, disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2-chlera)tricyclo[3.3.1.1 3,7 
9
4 Abbreviations used in this paper: Ii, invariant chain; CIITA, class II transactivator; CSPD, disodium 3-(4-methoxyspiro[1,2-dioxetane-3,2-chlera)tricyclo[3.3.1.1 3,7 
9

RT-PCR analysis

Total RNA was isolated from subconfluent cultures with TRIzol (Life Technologies) following the instructions of the manufacturer. cDNA preparation was performed on 1 μg total RNA with poly(dT) and 2.5 U Omniscript reverse transcriptase from Qiagen. PCR was performed with the Taq polymerase from Qiagen following the instructions of the manufacturer. Quantification of cDNA concentration was further assessed with GAPDH-specific primers through a 16-cycle PCR. The oligonucleotide primer set for CIITA (CTGACAGGTAGGACCCAGC) was used in association with the antisense CIITA-P-AS primer (TGCTGAACTGGTCGCAGTTGATGG). The sense primers, pIII-CIITA (GGAATTCCAGACTCCGGGAGCTGC) and pIV-CIITA (GCCAGAGCTGGCGGGAGGGA), were both used in association with pIII-initiated transcripts, 27 cycles were applied, and revealed with ready-to-use CSPD (Roche). The sense primers, pIII-CIITA (GGAATTCCAGACTCCGGGAGCTGC) and pIV-CIITA (GCCAGAGCTGGCGGGAGGGA), were both used in association with pIII-initiated transcripts, 27 cycles were applied, and revealed with ready-to-use CSPD (Roche). The sense primers, pIII-CIITA (GGAATTCCAGACTCCGGGAGCTGC) and pIV-CIITA (GCCAGAGCTGGCGGGAGGGA), were both used in association with pIII-initiated transcripts, 27 cycles were applied, and revealed with ready-to-use CSPD (Roche).

RACE-PCR

The RACE-PCR was performed with the 5’ RACE system from Life Technologies (Grand Island, NY) according to the manufacturer’s instructions. The cDNA preparation was performed with the CIITA-P-AS oligonucleotide described above, which hybridizes to all forms of CIITA transcripts. The antisense oligonucleotide specific for promoter III-initiated transcripts (CTGACAGGTAGGACCCAGC) was used in association with the anchored primer from the kit, with a 56°C annealing temperature and a 35-cycle amplification. The RACE-PCR products (200 pb) were directly subcloned in the PCR2.1 vector (TA-cloning kit from Invitrogen, San Diego, CA), and the inserts were sequenced using the Big Dye Terminator cycle sequencing kit (Perkin Elmer Applied Biosystems, Foster City, CA) using an ABI 377 automatic sequencer (Perkin-Elmer, Norwalk, CT).

Results

Coordinated HLA-D isotype expression at the cell surface of HLA-DR+ melanoma cell lines

Cell surface expression of HLA-DR molecules was analyzed on a panel of 20 different melanoma cell lines by cytofluorometry. As previously described by others (11, 12), two main phenotypes were obtained, with either no expression of HLA-DR in the absence of IFN-γ treatment (HLA-DR− cells), or constitutive expression of these molecules (HLA-DR+ cells). These phenotypes are exemplified in Fig. 1 for the 42/95 and HT144 cell lines. This study was further pursued, on four melanoma cell lines considered as prototypes in our study, with the analysis of all three HLA-D isotype expression, in the presence or the absence of IFN-γ (Table II). In two HLA-DR− cell lines, 42/95 and M74, none of the three isoforms was expressed in a constitutive manner. However, IFN-γ treatment induced the cell surface expression of HLA-DR and HLA-DP, with a null or very weak expression of HLA-DQ. Therefore, these melanoma cell lines have a pattern of MHC class II expression similar to fibroblast cell lines (BUA, Table II) or to epidermal melanocytes (19). With the HLA-DR+ cell lines, HT144 and A375, all three isoforms were expressed constitutively, with a further stimulation of expression following the IFN-γ treatment. Therefore, the deregulation of HLA-DR molecule expression in these melanoma cell lines is also affecting HLA-DP and HLA-DQ. However, constitutive and IFN-induced MHC class I expression did not differ significantly when HLA-DR+ and HLA-DR− cell lines were compared, thereby suggesting that the phenomenon is specific for the MHC class II molecules.

Constitutive expression of the HLA-D transcripts in the HLA-DR+ melanoma cell lines

The expression of the HLA-D transcripts was analyzed by RT-PCR experiments in the four melanoma cell lines. In the absence of a melanocyte cell line, we used as a control the BUA fibroblast cell line. As displayed on Fig. 2, constitutive expression of all the

<table>
<thead>
<tr>
<th>Amplified cDNA</th>
<th>Primer Set</th>
<th>Annealing Temperature (°C)</th>
<th>No. of Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>HLA-DRA</td>
<td>S</td>
<td>TGGGAGTTTGATGCCTCAAG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>AACATCATCACCTCCATGGT</td>
<td>58</td>
</tr>
<tr>
<td>HLA-DRB</td>
<td>S</td>
<td>CAGACATATAGGATGAGTCTCC</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTCCGACATCTGCTGCTCTCA</td>
<td>59</td>
</tr>
<tr>
<td>HLA-DQA</td>
<td>S</td>
<td>GTGCAGTCAGGTTGCAAATTTGACACG</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CACGAGATCGGATACAGCGGATAGCTG</td>
<td>59</td>
</tr>
<tr>
<td>HLA-DQB</td>
<td>S</td>
<td>CGGAACTCAGGAAYACCTCAAGAGG</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>GGAGTCATTTTACAGCATACAGAGG</td>
<td>58</td>
</tr>
<tr>
<td>HLA-DPA</td>
<td>S</td>
<td>GAGACCGCTCTGAGACCTCTGA</td>
<td>58</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTCTCAGGACACCTACAGT</td>
<td>59</td>
</tr>
<tr>
<td>HLA-DPB</td>
<td>S</td>
<td>GGGACAGCGCTCCTTCTCTGAG</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CAAAGGATGGTGGCTGCTGCA</td>
<td>54</td>
</tr>
<tr>
<td>HLA-DMB</td>
<td>S</td>
<td>GGGTACTCAGACCTTACCA</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CTTTCTCACCTGGAGTCTGA</td>
<td>65</td>
</tr>
<tr>
<td>GAPDH</td>
<td>S</td>
<td>TTTCTGGGGACCCCCTCCCCAC</td>
<td>62</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CACGGAGCTACCGCCGAGCA</td>
<td>63</td>
</tr>
<tr>
<td>CIITA</td>
<td>S</td>
<td>TCTCCCTCTTTCCACTGGAGTCTC</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>AS</td>
<td>CGGGGGGAAGTGGCTGAGA</td>
<td>62</td>
</tr>
<tr>
<td>IRF-1</td>
<td>S</td>
<td>TCTCCCTCTTTCCACTGGAGTCTC</td>
<td>63</td>
</tr>
</tbody>
</table>

* S, Sense; AS, antisense.
HLA-D mRNAs, in addition to Ii, was observed in the HT144 and A375 cell lines. In contrast, with the BUA and 42/95 cells, no expression of these genes was observed in the absence of IFN-γ treatment, except HLA-DPB and HLA-DQB transcripts detected in the untreated BUA cell line. The M74 cell line was more puzzling, as several genes displayed a constitutive expression (HLA-DRB, HLA-DQB, HLA-DMB, HLA-DPA, and HLA-DPB), even though HLA-DRA, HLA-DQA, and Ii mRNAs were not detected, even with a 30-cycle amplification RT-PCR procedure (data not shown). Therefore, the abnormal constitutive cell surface expression of the HLA-D molecules in the HLA-DR+ melanoma cell lines is in good agreement with a transcriptional deregulation of their encoding genes.

Luciferase gene reporter assays were next performed with HLA-DRA, HLA-DPA, and HLA-DQA promoter constructs in the HT144 and 42/95 cell lines. As depicted in Fig. 3, in the absence of IFN-γ treatment, luciferase activities driven by the HLA-D gene promoters were systematically greater (50- to 180-fold, depending on the promoter) in the HLA-DR+ HT144 cell line than in the HLA-DR− 42/95 cells. Therefore, these data show that the deregulation of the HLA-D molecules is caused by a factor activating the coordinate transcription of the HLA-D genes.

Interestingly, the IFN-γ stimulation of expression of the HLA-D promoter luciferase constructs in the HT144 cell line was very low. As an example, with the HLA-DRA promoter construct, stimulation following the IFN-γ treatment in the HT144 cell line was 1.4-fold, while it was 6-fold with the 42/95 cell line or 16-fold in the BUA cells (data not shown). The weak IFN-γ response correlates with the poor response to the cytokine previously observed in the analysis of most HLA-D transcripts in the HT144 cell line (Fig. 2).

CIITA is constitutively transcribed in both HLA-DR+ melanoma cell lines

As CIITA is the master switch of the tissue-specific expression pattern of the HLA-D molecules and of the accessory proteins implicated in Ag processing, we have next analyzed the expression of the CIITA mRNA in the four melanoma cell lines by RT-PCR (Fig. 4).

As previously described by others for fibroblast or melanoma cell lines (32), in the BUA and 42/95 cell lines, CIITA mRNA was not detected unless a 6-h IFN-γ treatment was applied, even though a 33-cycle PCR was applied on the cDNAs. In the HLA-DR+ M74 cell line, a very faint band was detected in the untreated cells. In contrast, in both HLA-DR+ cell lines, HT144 and A375, CIITA transcripts were observed even in the absence of the cytokine. CIITA transcription is inducible by IFN-γ in all these cell lines when using lower number of amplification cycles (see below). Therefore, the expression pattern of CIITA transcript was in agreement with the MHC class II phenotype, thereby indicating that the HLA-DR+ phenotype is highly likely caused by the abnormal constitutive expression of CIITA.

Analysis of the IFN-γ transduction pathway in HT144

Based on data cited above regarding the weak IFN-γ response observed in the HT144 cell line concerning the HLA-D genes, we

Table II. Summary of the data obtained by flow cytometry on the melanoma cell lines and on control cells, the B lymphocyte COM, and the fibroblast BUA cell lines*.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HLA-DR</th>
<th>HLA-DP</th>
<th>HLA-DQ</th>
<th>HLA-A,B,C</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>UNT</td>
<td>IFN-γ</td>
<td>UNT</td>
<td>IFN-γ</td>
</tr>
<tr>
<td>42/95</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>M74</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>BUA</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>COM</td>
<td>+++</td>
<td>ND</td>
<td>+++</td>
<td>ND</td>
</tr>
<tr>
<td>A375</td>
<td>++</td>
<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HT144</td>
<td>+++++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
</tr>
</tbody>
</table>

* Cells were either untreated (UNT) or treated for 72 h with IFN-γ, and indirect immunofluorescence analysis was performed with the L243, B7/21, L2, and W6/32 mAbs directed, respectively, against HLA-DR, HLA-DP, HLA-DQ, or HLA-A,B,C. The + and − symbols correspond to the following fluorescence means: −, 1–3; +, >3–20; ++, >20–100; +++, >100–400; +++++, >400.
hypothesized that the constitutive expression of CIITA might result from a permanent activation of the IFN-γ transduction pathway in this cell line, and might therefore lead to a poor additional response to the cytokine. Two IFN-γ-response genes were then analyzed for their transcription in the melanoma cell lines. IRF-1 is an early response gene induced by the binding of the phosphorylated STAT-1 heterodimer, and guanylate-binding protein 1 (GBP-1) transcription is occurring through IRF-1 activation (44). No major difference was detected between the HLA-DR\(^-\) and HLA-DR\(^+\) cell lines concerning the constitutive expression levels of IRF-1 (Fig. 5) or GBP-1 (data not shown) transcripts. The main difference between the cell lines is the kinetics of IFN-γ-response with IRF-1, whose transcript accumulation is reduced in the A375 and 42/95 cell lines after a 48-h treatment compared with the other melanoma cell lines. Therefore, these data indicate that the general signal transduction mediated by the cytokine is similar in both HLA-DR\(^-\) and HLA-DR\(^+\) melanoma cell lines.

**Comparable basal level of CIITA transcription initiated from promoter IV in both melanoma cell lines**

In cells such as fibroblasts or HLA-DR\(^-\) melanoma, the IFN-γ-induced transcription of CIITA is mainly initiated from promoter IV (32). By semiquantitative RT-PCR, we have analyzed the initiation of CIITA transcripts from promoter IV in the melanoma cell lines, compared with the fibroblast BUA cell line. As the sequence of exon 1 differs depending on the promoter used, we chose a sense primer specific of exon 1 transcribed from promoter IV and an antisense primer hybridizing to the exon 2 common to all mRNA initiated from the different CIITA promoters. As depicted in Fig. 6, in the absence of IFN-γ-treatment, transcription initiated from promoter IV is not observed in the melanoma and in the fibroblast cell lines. However, with the HT144 and A375 cells, in the absence of IFN-γ-treatment, a very faint band was detected when the film was overexposed. This barely detectable band did not reflect the level of transcription obtained in the preceding experiment, and these data suggested that the HLA-DR\(^+\) phenotype was not explained by a constitutive expression from promoter IV of CIITA.

These results were confirmed by luciferase gene reporter assays performed with a CIITA promoter IV construct transfected in the HT144, A375, and 42/95 melanoma and the fibroblast BUA cell lines (Fig. 7). In three independent experiments, we have never observed more than a 2-fold difference in the constitutive luciferase amounts in the HLA-DR\(^+\) cells compared with the HLA-DR\(^-\) and BUA cell lines. Concerning IFN-γ induction of the CIITA promoter IV, the induction was slightly higher in the BUA cell line (10-fold), compared with the HT144, A375, and 42/95 cell lines (~8-fold). In the HLA-DR\(^-\) M74 cell line, stimulation by IFN-γ was ranging from 20- to 40-fold (not shown) with promoter IV constructs, which is in good agreement with the high induction of CIITA transcripts from promoter IV (Fig. 6). These results further confirm the absence of deregulation of the IFN-γ transduction pathway in the HLA-DR\(^+\) cell lines and a normal usage of CIITA promoter IV in all these cell lines.

**Constitutive transcription of CIITA in HT144 melanoma cell line mainly initiates from promoter III**

We had previously shown that the lack of constitutive expression of CIITA in epithelioid cells was due to an active suppression phenomenon of CIITA promoter III (45). Therefore, we hypothesized that the suppression mechanism might be inactivated in the HLA-DR\(^+\) cell lines, and that the constitutive transcription of CIITA might be initiated from the promoter III of this gene. RACE-PCR was then performed with an antisense primer hybridizing to the common exon 2 shared by all CIITA mRNA, and the product was further amplified using a primer specific for promoter III-initiated transcripts. In the HT144 cell line, a band was amplified in untreated cells, and its intensity increased in cells treated for 6 h with IFN-γ (data not shown). This band presented the same sequence that was previously described on mRNA initiated from promoter III in B lymphocytes (28, 32).

We further confirmed this result by a 25-amplification cycle semiquantitative RT-PCR using a sense primer specific for promoter III-initiated transcript, and the COM B lymphocyte cell line as a positive control. As expected, a high level of CIITA mRNA was initiated from promoter III in COM (Fig. 8). In the absence of IFN-γ, the 42/95 cell line did not display any CIITA transcript even in experiments using 33-cycle PCR amplification (data not shown). In contrast, in the HLA-DR\(^+\) HT144 and A375 cell lines, CIITA product was observed in the absence of the cytokine. In the M74 cell line, and in the BUA cell line, a faint band was detected, which is in agreement with the constitutive transcription of certain HLA-D transcripts that was observed previously (Fig. 2). After IFN-γ treatment, all cell lines displayed an increase of CIITA transcription from promoter III, in agreement with the presence of the IFN-responsive enhancer located upstream of promoter III (34, 36).

In conclusion, the constitutive expression of CIITA in the HLA-DR\(^+\) cell lines is due to an abnormal transcription initiated from promoter III that was described to be mainly used in B lymphocytes.

**The initiation from promoter III of CIITA in HT144 requires the presence of the IFN-γ-responsive enhancer located upstream promoter III**

To determine whether the deregulation of CIITA transcription was due to a trans-acting factor, luciferase reporter gene assays were performed with different promoter III enhancer constructs depicted in Fig. 9.

With promoter III constructs alone (either 545 bp or 1723 bp long), the difference between the 42/95 and HT144 cell lines did not exceed 1.5-fold in four independent experiments. However, constructs containing the enhancer were driving a luciferase activity 7- to 9-fold higher in the HT144 cell line than in the 42/95 cells, these values representing an average of five independent experiments. Therefore, our data show that the 1-kb enhancer, previously described as an IFN-responsive element in epithelioid cell lines, is the target for CIITA constitutive expression in melanoma cell lines.

Interestingly, the properties of the enhancer in the HT144 cell line were lost when the SV40 promoter (Fig. 10, pGL3-SV40.Enh construct) replaced the minimal promoter III of CIITA. These data suggest that the factor(s) binding the enhancer additionally requires an interaction with the CIITA promoter III, or with promoter III-binding factor(s), for a complete activity.

**Analysis of the Brn-2 transcription factor**

Brn-2/N-Oct3 has been described as a transcription factor involved in the tumorigenic potential of melanoma cells (46). In addition, it was shown that transfection of its encoding cDNA activates the transcription of MHC class II genes (47). As a perfect consensus binding site sequence (CATGCAAATG) was found in the 1-kb enhancer (43), Brn-2 represented a good candidate for the deregulation of CIITA transcription. We first did not evidence any major difference in the amount of its mRNA or protein content when comparing 42/95 and HT144 cell lines (data not shown). However, description of posttranslational events modulating Brn-2 activity (48) led us to assay the effect of a deletion (pIIDEL2.Brn-Del.Luc construct) or a mutation (pIIDEL2.Brn-Mut.Luc construct) in the
FIGURE 3. Activity of the HLA-DRA, HLA-DQA, and HLA-DPA gene promoters measured by luciferase assays in the HT144 and 42/95 cell lines. After correction of transfection efficiency with β-galactosidase, the data were expressed as the ratio of RLUs⁻¹ obtained in the HT144 cell line divided by the RLUs⁻¹ obtained in the 42/95 cells. These data represent the average of four independent experiments.

Brn-2 palindromic site created in the pIIDEL2.CIITA.Luc plasmid. As seen on Fig. 11, however, even though the mutation provoked a slight decrease on CIITA promoter III activity in both 42/95 and HT144 cell lines, we did not evidence any major effect of the Brn-2 binding site mutations neither in the 42/95 nor the HT144 melanoma cell lines.

In conclusion, whereas the 500-bp minimal promoter III is sufficient in B lymphocytes for constitutive expression of CIITA, the abnormal constitutive expression of CIITA in HT144 requires the additional presence of the 1-kb enhancer element located upstream promoter III, and deregulation of CIITA is mediated by a trans-acting factor in the melanoma cell line.

Discussion
Our data demonstrate that the constitutive expression of MHC class II molecules in melanoma is correlated with a constitutive transcription of CIITA. This phenomenon was observed in two HLA-DR⁺ cell lines established from unrelated patients, thereby suggesting that it might not originate from a random mutagenesis event, but might be linked to the neoplastic fate or differentiation state of this tumor type.

We have shown that the deregulation of CIITA in melanoma cells occurs through the 1-kb enhancer upstream of promoter III, which was previously described as an IFN-responsive sequence (34, 36). Our study has evidenced that the basal expression level of different IFN-responsive genes (MHC class I, GBP-1, or IRF-1) is similar in both HLA-DR⁺ and HLA-DR⁻ melanoma cell lines (Table II and Fig. 5). This demonstrates that the constitutive expression of CIITA transcript is not caused by a general deregulation of the IFN transduction pathway. However, A375 and HT144 cells are poorly inducible by this cytokine for the expression of the HLA-D genes (Fig. 2). Based on the above data, this observation might be explained by a quantitative effect of CIITA. Indeed, it was shown that over a certain threshold of CIITA, MHC class II expression reached a plateau (49). In the HT144 and A375 cell lines, CIITA mRNA levels are constitutively high (Fig. 8) and allow an HLA-DR expression that is quite similar to that obtained with the induced BUA fibroblast cell line (Table II). When the HLA-DR⁺ cell lines are induced by IFN-γ, the CIITA transcription, initiated from both promoters III and IV (Figs. 6 and 8), does not result in a drastic increase of HLA-D transcription, as CIITA is already in high amounts before the addition of the cytokine. Two results are interesting to point out concerning CIITA transcription. Even though luciferase expression driven by CIITA promoter IV has a comparable basal expression level in HLA-DR⁺ and HLA-DR⁻ melanoma cell lines (Fig. 7), a low amount of promoter IV-initiated transcripts was detected in the HLA-DR⁻ melanoma cell lines by RT-PCR (Fig. 6). This suggests that the enhancer/promoter III endogeneous sequences might trans-activate the adjacent promoter IV. The second point is the fact that the enhancer of CIITA did not function when associated with a heterologous promoter, neither in the HT144 cell line concerning its constitutive expression level (Fig. 10), nor in a fibroblast cell line after an IFN-γ treatment (J. Vedrenne, unpublished data).

Luciferase assays reported in this work demonstrated that constitutive expression of CIITA transcription occurs via trans-acting factor(s) binding on the enhancer in the HLA-DR⁺ melanoma cell lines. As a putative candidate, Brn-2, was dismissed in this study, a random mutagenesis of the enhancer will be required to identify the factor activating the constitutive expression of CIITA. This factor might either be a suppressor inactivated during the tumorigenesis of the melanoma cells, or an activator gaining transcriptional activity or binding capacities to the CIITA enhancer. As this factor interacts with the enhancer, our data show that it is different from the suppressor binding on promoter III of CIITA evidenced in epithelial cells (45). Interestingly, the HLA-DR⁻ M74 cell line is displaying a faint constitutive expression of certain HLA-D genes and a weak CIITA promoter III-initiated transcription relative to the HLA-DR⁺ cell lines. This suggests that the factor interacting with the enhancer might have different levels of expression or activation depending on the melanoma cell line.
In many nonprofessional APC, cell surface expression of HLA-DQ is weak or null even when treated with IFN-γ (reviewed in Ref. 50). This phenomenon was observed as well with the HLA-DR− melanoma cell lines, in which the lack of expression of cell surface HLA-DQ molecules is obviously explained by the absence of HLA-DQA transcripts, even after a 48-h IFN-γ treatment, although HLA-DQB transcript is induced by the cytokine (Fig. 2). However, the HLA-DQA promoter is functional, as the transfection of the CIITA cDNA in the 42/95 cell line restores cell surface expression of the HLA-DQ molecules (data not shown). In addition, in this cell line, we showed that an exogenous HLA-DQA promoter is able to drive the expression of a luciferase reporter gene (Fig. 3). This suggests the presence of a dose-limiting repressor acting on this promoter, whose effect would be bypassed in the luciferase assays by the high number of HLA-DQA promoter sequences carried by the plasmid constructs. Indeed, a HLA-DQA-specific binding factor has been described as interacting with the Y box (51), and might possess a repressing activity specific for the HLA-DQA promoter. An alternative hypothesis was given by Otten et al. (49), who demonstrated that cell surface HLA-DQ expression required a higher dose of CIITA relative to the expression of HLA-DR and HLA-DP. As HLA-DR+ cells, expressing CIITA constitutively, also express HLA-DQ, it could be proposed that a threshold of CIITA protein must be crossed to detect HLA-DQ expression. However, this hypothesis is not fully satisfying with the melanoma cell lines. Indeed, in the M74 cell line treated with IFN-γ, cell surface HLA-DQ expression is not observed, and HLA-DQA transcripts are not detected. However, in this cell line, the amount of CIITA transcripts initiated from promoter IV is even higher than in the HLA-DR+ cell lines (Fig. 6). This could indicate that the transcription of the HLA-DQA gene requires the presence of promoter III-initiated CIITA transcripts that are in low amounts in both HLA-DR− melanoma cell lines treated with IFN-γ (Fig. 8).

With CIITA transcripts initiated from promoter III, the CIITA protein can be translated from two different ATGs (28). Therefore, it is possible that the longer form of CIITA (initiated from the first ATG) alone might be able of trans-activating the HLA-DQA gene. It might be more active than the shorter form of CIITA or be more efficient concerning the removal of an HLA-DQA-specific repressor. Indeed, we have observed a longer form of the CIITA protein in the HT144 cell line compared with the 42/95 cell line (G. Barbieri, manuscript in preparation).

The last point we address in this work is the role of the constitutive expression of MHC class II molecules in melanoma, as this expression is considered as a progression marker (15–17). Tumor Ags can be presented via the HLA-DR molecules in melanoma cell lines (8), and the lack of costimulatory molecules might lead to tumor-specific T lymphocyte anergy. A second hypothesis is that the constitutive expression of CIITA might enhance cell growth. However, in our hands, CIITA overexpression in transfected cells tends to slow down the cell growth (our unpublished observations). We alternatively propose that the factor deregulating CIITA transcription might confer a selective advantage to the tumor cells. Finally, CIITA might activate the expression of genes that could facilitate tumor invasiveness or immune escape. In agreement with this hypothesis, CIITA was recently shown as able to repress the expression of Fas ligand in T lymphocytes (52). A closer analysis

FIGURE 7. Activity of the promoter IV of CIITA measured by luciferase assays. Cells were either untreated (□) or treated with 250 U/ml IFN-γ for 24 h (■). Transfection efficiency was corrected with β-galactosidase, and these data represent the average of three independent experiments.

In many nonprofessional APC, cell surface expression of HLA-DQ is weak or null even when treated with IFN-γ (reviewed in Ref. 50). This phenomenon was observed as well with the HLA-DR− melanoma cell lines, in which the lack of expression of cell surface HLA-DQ molecules is obviously explained by the absence of HLA-DQA transcripts, even after a 48-h IFN-γ treatment, although HLA-DQB transcript is induced by the cytokine (Fig. 2). However, the HLA-DQA promoter is functional, as the transfection of the CIITA cDNA in the 42/95 cell line restores cell surface expression of the HLA-DQ molecules (data not shown). In addition, in this cell line, we showed that an exogenous HLA-DQA promoter is able to drive the expression of a luciferase reporter gene (Fig. 3). This suggests the presence of a dose-limiting repressor acting on this promoter, whose effect would be bypassed in the luciferase assays by the high number of HLA-DQA promoter sequences carried by the plasmid constructs. Indeed, a HLA-DQA-specific binding factor has been described as interacting with the Y box (51), and might possess a repressing activity specific for the HLA-DQA promoter. An alternative hypothesis was given by Otten et al. (49), who demonstrated that cell surface HLA-DQ expression required a higher dose of CIITA relative to the expression of HLA-DR and HLA-DP. As HLA-DR+ cells, expressing CIITA constitutively, also express HLA-DQ, it could be proposed that a threshold of CIITA protein must be crossed to detect HLA-DQ expression. However, this hypothesis is not fully satisfying with the melanoma cell lines. Indeed, in the M74 cell line treated with IFN-γ, cell surface HLA-DQ expression is not observed, and HLA-DQA transcripts are not detected. However, in this cell line, the amount of CIITA transcripts initiated from promoter IV is even higher than in the HLA-DR+ cell lines (Fig. 6). This could indicate that the transcription of the HLA-DQA gene requires the presence of promoter III-initiated CIITA transcripts that are in low amounts in both HLA-DR− melanoma cell lines treated with IFN-γ (Fig. 8).

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FIGURE 8. RT-PCR analysis of promoter III-initiated CIITA transcripts. Twenty-five-cycle and 16-cycle amplification procedures were applied, respectively, for CIITA and GAPDH transcripts, and Dig-labeled products were stained with CSPD.

FIGURE 9. Luciferase assays with different combinations of the promoter III and enhancer sequences of CIITA. After correction of transfection efficiency with β-galactosidase, the data were expressed as the ratio of RLUs−1 obtained in the HT144 cell line divided by the RLUs−1 obtained in the 42/95 cells. These experiments correspond to an average of at least four independent experiments.

FIGURE 10. Luciferase assays performed in the HT144 cell line using different constructs whose structure is detailed at the bottom of the figure. The experiments were performed in triplicate, using either a β-galactosidase (upper figure) or a Renilla (lower figure) reporter gene vector to control for transfection efficiency.
of genes activated or repressed by CIITA must therefore be undertaken, as this protein might well interact with a much broader number of target genes than previously thought.

We and others have previously reported that different tumor cell lines display MHC class II alteration of expression, and that CIITA is the direct or indirect target of these alterations (53, 54). The analysis of such cells will help to gain further insights in CIITA expression and function, and thereby in MHC class II gene regulation. In addition, these studies might help in the understanding of the mechanisms involved in the escape of tumors from the immune system.

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We thank E. Tartour for the melanoma cell lines, E. Benveniste for the expression and function, and thereby in the analysis of such cells will help to gain further insights in CIITA expression and function, and thereby in MHC class II gene regulation. In addition, these studies might help in the understanding of the mechanisms involved in the escape of tumors from the immune system.

References


