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Linkage between STAT Regulation and Epstein-Barr Virus Gene Expression in Tumors

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Epstein-Barr virus (EBV) latency gene expression in lymphoblastoid cell lines is regulated by EBNA2. However, the factors regulating viral expression in EBV-associated tumors that do not express EBNA2 are poorly understood. In EBV-associated tumors, EBNA1 and frequently LMP1 are synthesized. We found that an alternative latent membrane protein 1 (LMP1) promoter, L1-TR, located within the terminal repeats is active in both nasopharyngeal carcinoma and Hodgkin's disease tissues. Examination of the L1-TR and the standard ED-L1 LMP1 promoters in electrophoretic mobility shift assays revealed that both promoters contain functional STAT binding sites. Further, both LMP1 promoters responded in reporter assays to activation of JAK-STAT signaling. Cotransfection of JAK1 or v-Src or treatment of cells with the cytokine interleukin-6 upregulated expression from ED-L1 and L1-TR reporter plasmids. Cotransfection of a dominant negative STAT3β revealed that STAT3 is likely to be the biologically relevant STAT for EBNA1 Qp and LMP1 L1-TR promoter regulation. In contrast, LMP1 expression from ED-L1 was not abrogated by STAT3β, indicating that the two LMP1 promoters are regulated by different STAT family members. Taken together with the previous demonstration of JAK-STAT activation of Qp driven EBNA1 expression, this places two of the EBV genes most commonly expressed in tumors under the control of the same signal transduction pathway. Immunohistochemical analyses of nasopharyngeal carcinoma tumors revealed that STAT3, STAT5, and STAT1 are constitutively activated in these tumors while STAT3 is constitutively activated in the malignant cells of Hodgkin's disease. We hypothesize that chronic or aberrant STAT activation may be both a necessary and predisposing event for EBV-driven tumorigenesis in immunocompetent individuals.

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that, after primary exposure, maintains a latent infection for the life of the individual. Approximately 1 to 50 per 10^6 circulating B cells in healthy seropositive individuals carry the EBV genome, and the site of long-term latency has been identified as the G_0 memory B cell (39). EBV infection elicits a strong immune response (44), and in general viral persistence is controlled by the host and is asymptomatic. However, one consequence of lifelong infection is the potential for the development of EBV-associated malignancies, which include Burkitt's lymphoma, nasopharyngeal carcinoma (NPC), Hodgkin's disease, lymphoproliferative disease in immunocompromised patients, primary central nervous system lymphoma in AIDS patients, nasal T-cell lymphoma, a subset of gastric carcinoma, and possibly also a subset of primary liver and breast cancers (2, 3, 43, 51).

On initial EBV infection, and in latently infected lymphoblastoid cell lines in culture, the full spectrum of EBV latency genes is expressed. The Wp promoter, which is regulated by B-cell-specific factors, is responsible for the initial transcription of the nuclear EBNAs, EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP (32). EBNA2, which functions as a transcriptional activator, then enforces a switch to Cp promoter-driven EBNA synthesis and also regulates synthesis of the LMP1 and LMP2 latency membrane proteins. However, EBNA2 is detected in EBV-associated malignancies only in the context of immunosuppression, presumably because the Cp-driven EBNA3 family proteins elicit a robust CD8 cell-mediated immune response (44). In EBV-associated tumors in immunocompetent patients, the Cp is repressed by methylation (1, 41). An alternative TATA-less promoter, Qp (40, 46), is used to express EBNA1 in the absence of the immunogenic EBNAs, and LMP1 is also frequently expressed. EBNA1 binds to the origin of latent DNA replication, oriP, and is required for maintainance of the episomal form of the latent EBV genome (35, 42, 60). LMP1, an integral membrane protein, is essential for EBV-driven B-cell immortalization and induces transformation in primary Rat1 cells (56). The transforming ability of LMP1 is explicable in large part by its functioning as a constituitively activated tumor necrosis factor (TNF) receptor that mimics signaling by the B-lymphocyte activation antigen CD40 (19, 24, 55). The cytoplasmic carboxy terminus of LMP1 interacts with TNF-receptor associated factors and with the TNF receptor-associated death domain protein to activate NF-κB and JNK (c-Jun N-terminal kinase) signaling (17, 29).

It has been unclear how EBV gene expression in tumors is regulated in the absence of EBNA2. We recently provided evidence that the EBNA1-Qp promoter contains binding sites for STATs and is activated in transient expression assays by stimulation of JAK (Janus kinase)-STAT (signal transducer

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and activator of transcription) signaling (12). The JAK-STAT pathway transduces signals from receptor-bound cytokines and growth factors to the nucleus (4, 47). Ligand-induced receptor aggregation leads to autophosphorylation of the receptor-associated JAKs followed by tyrosine phosphorylation of the receptor. The receptor phosphotyrosines serve as docking sites for the SH2 domain of STAT monomers, which are then themselves tyrosine phosphorylated either directly by JAKs or by other nonreceptor protein tyrosine kinases such as c-Src (47). The STAT family of proteins consists of seven members that reside in the cytoplasm. Tyrosine phosphorylation leads to SH2-mediated homo- or heterodimerization and translocation to the nucleus, where the STATs bind to their target DNA recognition sequences and activate transcription. STAT transcriptional activity is further modulated by phosphorylation of a critical serine residue in the transactivation domain. Mitogen-activated protein kinases (MAPK), JNK, and protein kinase C have been implicated in serine phosphorylation of different STAT family members (4). In normal cell signaling events, STAT activation is transient. Negative regulation is produced by dephosphorylation of signaling intermediates by protein tyrosine phosphatases, by the SOCS family of JAK inhibitors, and by the induction of STAT inhibitors such as PIAS (protein inhibitor of activated STAT) (47, 50).

Several oncogenes function by constituitively activating STAT signaling. A well-characterized example is v-Src, which induces constituitive tyrosine phosphorylation of STAT3, STAT5, and STAT1 (11, 61, 62). The association between aberrant STAT activation and v-Src oncogenic activity was strengthened by the demonstration that constituitive activation of STAT3 by modification of the SH2 domain results in a protein that is able to induce cells to form colonies in soft agar and tumors in nude mice (5, 15). Aberrant activation of JAK-STAT signaling has also been described in human cancers. The chromosomal translocation that gives rise to the Tel-JAK2 fusion protein in acute lymphocytic leukemia creates an aberrantly activated JAK2 kinase by forced dimerization of the kinase through the dimerization domain of the Ets protein fusion partner (9). In addition, the tumor cells in malignancies such as breast cancer, head and neck cancers, and a variety of leukemias and lymphomas contain increased levels of activated nuclear STATs, frequently STAT3 or STAT5 (4, 23, 57, 58).

To assess the contribution of STAT signaling to EBV latency gene expression, we examined LMP1 transcription and found that both the standard LMP1 promoter and an alternative LMP1 promoter located further upstream in the viral terminal repeats are STAT responsive. Thus, two EBV genes commonly expressed in tumors, EBNA1 and LMP1, are regulated by this pathway. Further, an immunohistochemical analysis of samples from patients with the EBV-associated malignancies NPC and Hodgkin's disease identified nuclear activated STATs within tumor cells. We believe that STATs, and in particular STAT3, are a driving force for EBV gene expression in tumors and suggest that disregulation of the JAK-STAT pathway may be a predisposing event for EBV-associated tumorigenesis.

MATERIALS AND METHODS

Cells and tissues. The JAK1 mutant (U4A), TYK2 mutant (U1A), and wild-type parental (2fTGH) cell lines were a generous gift of G. Stark (34). These cells were maintained in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum and hygromycin (250 μg/ml). The EBV-positive B-cell line B95-8 was grown in RPMI 1640 plus 10% fetal bovine serum. Induction with interleukin-6 was achieved by incubating B95-8 cells in medium plus 100 ng of human interleukin-6 (IL-6) (R&D Systems, Minneapolis, Minn.) per ml for 48 h before harvesting the cells for RNA extraction. NPC tissues and paraffin sections were obtained from Queen Mary Hospital, Hong Kong, and Sun Yat-Sen Medical University Tumor Hospital, Guangzhou, China, respectively. Hodgkin's disease samples were obtained from the Department of Pathology, Johns Hopkins Hospital.

Plasmids. The LMP1-chloramphenicol acetyltransferase (CAT) reporter pLRS324 was obtained from L. Rymo (21). L1TRp-CAT and L1TR(mt)p-CAT reporters were constructed in pCAT-BASIC (Promega). L1TRp-CAT contains DNA sequences from coordinates 169981 to 170317 of the EBV genome. In L1TR(mt)p-CAT, core sequences of the STAT binding site were mutated (TT CCTGGAA to ggaCTcGtg). The Qp-CAT reporter expresses CAT from the EBV latency Q promoter and has been previously described (12). Expression plasmids for STAT3β and v-Src were gifts from R. Jove (6, 54), and the JAK1 expression vector has been described previously (12).

CAT assays. HeLa cells were transfected using the calcium phosphate procedure (12), and U1A, U4A and 2fTGH cells were transfected using SuperFectant (Qiagen) as specified by the manufacturer. Cells were transfected with 1 or 2 μg of reporter DNA and 1 or 2 μg of effector DNA, and total transfected DNA was equalized using vector DNA. Cells were harvested 30 to 40 h after transfection. Reporter activity was quantitated as previously described (12) using an Instant-Imager (Beckman Instruments).

Electrophoretic mobility shift assay. Purified, activated STAT1 and STAT4 proteins were generous gifts of T. Hoey, Tularik, Calif. (59). The sequence of the sense strand of the double-stranded DNA probes and competitor oligonucleotides are as follows, with introduced mutations shown in lowercase: 5′-CATGT TATGCATATTCTTGTAAGTGCATG (STAT1), 5′-GAGCTTGATTTCCCC GAAATGATGAGCGATC (STAT4), 5′-GATCGGGGGCCGCGCATTCCTG GAAAAAGTGGAGGG (L1TR), 5′-GATCGGGGGCCGCGCAggaCTcGtG AAAGTGGAGGG [L1TR(mt)], and 5′-GATCCGGGTACAGATTTCCCGA AAGCGGCGGTG (ED-L1). The Qp and control Flag oligonucleotides and the electrophoretic mobility shift assay (EMSA) conditions were as previously described (12). Anti-STAT4 and anti-Flag polyclonal antibodies used for supershift assays were obtained from Santa Cruz, Santa Cruz, Calif., and Sigma, St. Louis, Mo., respectively.

Northern and RT-PCR analyses. RNA was prepared from fresh NPC biopsy specimens as previously described (14). Oligo(dT)-enriched RNA (5 μg) was fractionated by agarose-formaldehyde gel electrophoresis, transferred to a Hybond N membrane (Amersham), and probed with a ^{32}P-labeled *Bam*HI Nhet EBV DNA fragment. RNA from B95-8 cells and Hodgkin's disease tissue were prepared using the QuickPrep Micro RNA purification kit (Pharmacia-Amersham) and amplified by reverse transcription PCR (RT-PCR) using the L1TR primers (position 168928) 5′-GCAGATTACACTGCCGCTTC and (position 169831) 5′-CCAGAGCATCTCCAATAAGTAG. PCR products were visualized by Southern blotting using a ^{32}P-end-labeled LMP1 oligonucleotide probe, (position 169455) 5′-CTCTCAAGGTCGTGTTCCATC. Oligonucleotides for EBER1 amplification and detection were as described previously (13).

Immunohistochemistry. Paraffin sections of NPC and Hodgkin's disease tissue were analyzed for STAT expression using anti-STAT1, anti-STAT5, and anti-STAT3 primary antibodies (Santa Cruz) at a 1:300 dilution. Positive interactions were visualized using the StrpABComplex/horseradish peroxidase Duet kit (Dako) as specified by the manufacturer. In control samples, the primary antibodies were replaced with normal rabbit serum. Samples were counterstained with hematoxylin.

RESULTS

Alternative LMP1 promoter usage in tumor samples. EBV LMP1 is essential for in vitro immortalization of B lymphocytes and is expressed in EBV-associated malignancies such as NPC and Hodgkin's disease (32, 43). The ED-L1 promoter is used to express LMP1 in B lymphoblastoid cell lines, but an alternative promoter located within the viral terminal repeats, L1-TR, has been described in NPC (45, 53). L1-TR is located approximately 600 bp upstream of ED-L1 (Fig. 1a) and gives rise to a larger (3.5-kb) transcript compared to the 2.8-kb ED-L1-initiated mRNA. The two transcripts use the same ATG

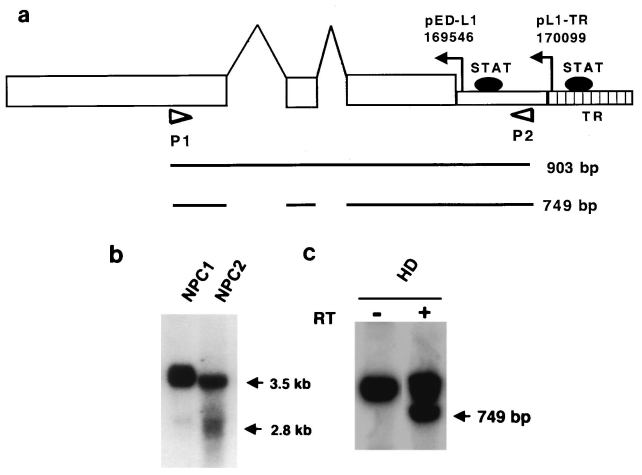

FIG. 1. LMP1 promoter usage in EBV-associated tumors. (a) Diagram of the LMP1 gene, showing the exon structure and the relative positions of the standard promoter (pED-L1) and the terminal repeat (TR) promoter previously identified in NPC tissue samples (pL1-TR) (45,53). The locations of potential STAT binding sites and of the PCR primers (P1 and P2) used to detect pL1-TR initiated mRNAs are also indicated. (b) Northern blot analysis of LMP1 mRNA isolated from two NPC tissue biopsy specimens. A 3.5-kb RNA indicative of pL1-TR usage was detected in both samples. A 2.8-kb RNA initiating from pED-L1 was also seen in NPC2. (c) Southern blot analysis of RT-PCR products generated using the P1 and P2 primers and RNA isolated from a case of EBV-positive Hodgkin's disease. The blot was incubated with an LMP1-specific ^{32}P-labeled oligonucleotide probe. The 749-bp product is diagnostic for an LMP1 mRNA initiating from pL1-TR. The 903-bp product was also detected in the absence of the RT reaction and was generated from EBV genomic DNA.

initiator, and hence the encoded LMP1 protein is identical. Transcription from the L1-TR promoter in NPC tumor tissue is illustrated in Fig. 1b, in which Northern blot analysis detected the 3.5-kb LMP1 mRNA. Low levels of the ED-L1-initiated 2.8-kb LMP1 transcript were also observed in one of the NPC samples (Fig. 1b). LMP1 expression is particularly prominent in EBV-positive Hodgkin's disease (30), and we therefore examined a sample of Hodgkin's disease tissue for L1-TR promoter usage. RT-PCR was performed using the P1 and P2 primers (Fig. 1a), which specifically detect L1-TR-initiated transcripts. A 749-bp product that reacted with an LMP1-specific oligonucleotide probe was detected by Southern blotting (Fig. 1c), indicating L1-TR usage in EBV-positive Hodgkin's disease. (The larger product was detected in the absence of the RT reaction and is amplified from EBV genomic DNA.)

The ED-L1 and L1-TR LMP1 promoters bind STATs. The ED-L1 promoter is complexly regulated and responds to EBNA2 in lymphoblastoid cell lines. However, EBNA2 is not expressed in EBV-associated tumors in immunocompetent hosts, suggesting a greater role for cellular factors in this setting. The activity of the TATA-less L1-TR promoter is modulated by the Sp1 and Sp3 transcription factors (45, 53). We recently demonstrated that the EBV Qp promoter that drives EBNA1 expression is activated by the JAK-STAT signaling pathway. We were intrigued by the possibility that this pathway might play a central role in the regulation of EBV latency gene expression in tumors, and examination of the ED-L1 and L1-TR sequences revealed potential STAT binding sites in each promoter (Table 1).

STATs dimerize, enter the nucleus, and bind to DNA when they are activated by tyrosine phosphorylation. STAT family proteins recognize similar DNA sequences, and it is not possible to predict which STATs might bind to the LMP1 promoters. We had available purified, activated STAT1 and STAT4 and used these reagents to test whether the LMP1 promoters contained functional STAT binding sites. EMSAs were performed using ^{32}P-labeled oligonucleotide probes containing ED-L1 and L1-TR sequences (Fig. 2). STAT1 bound strongly to both the ED-L1 and L1-TR probes but did not bind to the latency Qp probe. STAT4 also bound to the ED-L1 and L1-TR probes and, less strongly, to the Qp probe (Fig. 2a). To establish the specificity of the STAT binding, competition and supershift assays were performed using the LMP L1-TR probe and purified, activated STAT4 (Fig. 2b). The STAT4 complexes were competed away by excess unlabeled STAT4 binding-site oligonucleotide but not by an irrelevant olignucleotide containing the Flag epitope (Flag). Binding was also competed effectively by unlabeled L1-TR oligonucleotide but not by an L1-TR oligonucleotide carrying mutations within the STAT binding sequence (L1-TRmt). Addition of anti-STAT antibody generated a supershifted complex. No supershifted complex was formed using a control antibody (anti-Flag). These results demonstrate that the STAT sequences in the LMP1 promoters are functional binding sites. It should be noted that different STATs recognize very similar binding sites. The in vitro binding by STAT1 and STAT4, while demonstrating that these STATs are capable of binding to the EBV latency promoters, does not necessarily mean that they will be the STAT family members that regulate the promoters in vivo.

Activation of the L1-TR LMP1 promoter by the JAK-STAT pathway. Cytoplasmic STATs are tyrosine phosphorylated by JAKs as part of a signaling pathway that is initiated by cytokine or growth factor binding at the cell surface. The JAK protein tyrosine kinase family consists of JAK1, JAK2, JAK3, and TYK2. To test the L1-TR promoter for responsiveness to JAK-STAT signaling, transient-expression assays were performed using an L1-TR promoter-CAT reporter. Cotransfection of L1-TRp–CAT with a JAK1 expression vector substantially increased reporter activity over that observed in cells transfected with L1-TRp–CAT alone. IL-6 is a cytokine that influences B and T-cell growth and differentiation and is an important mediator of acute-phase immune responses (27). The IL-6 receptor signals through JAK1, JAK2, and TYK2, and the signaling activates STAT3 and STAT1 (47). Treatment of transfected cells with IL-6 also increased L1-TRp–CAT reporter activity (Fig. 3a).

Consistent with the observed increase in L1-TRp–CAT expression upon activation of JAK-STAT signaling, loss of JAK activity led to a corresponding decrease in reporter activity

TABLE 1. STAT binding sites in LMP1 and EBNA1 promoters

EBV promoter	STAT site
EBNA1 (Qp)	TT GCGAA AA
LMP1, (L1-TR)	TT CCTGG AA
LMP1 (ED-L1)	TT TCCCG AA

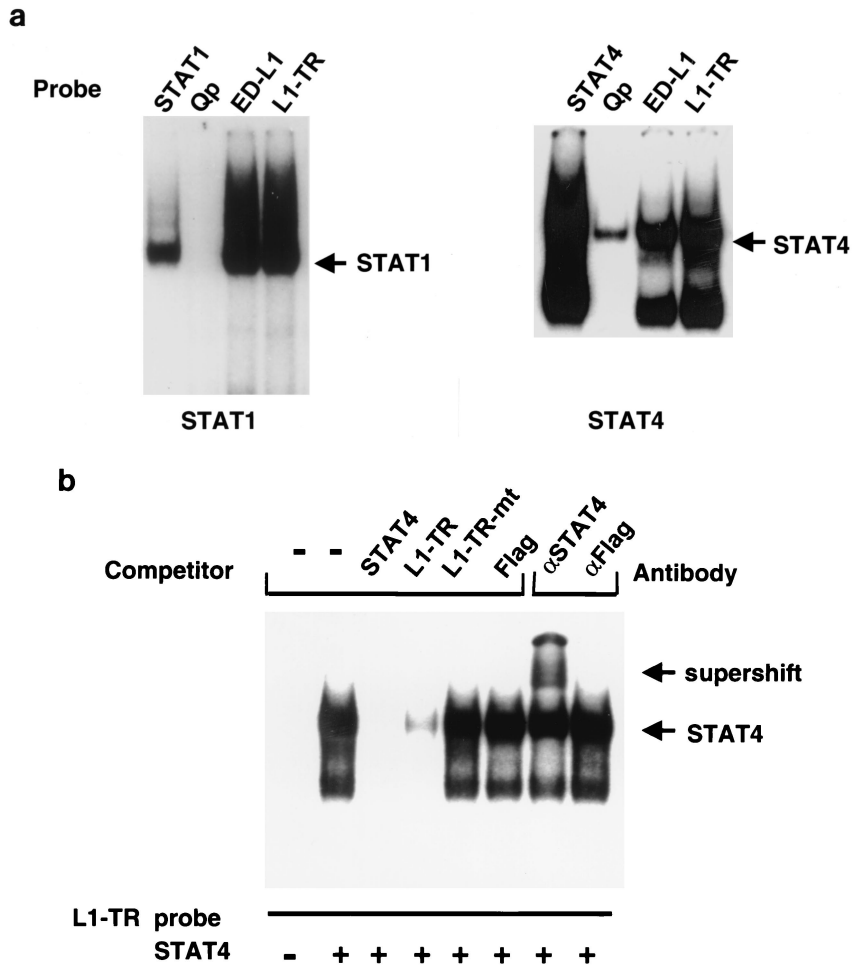

FIG. 2. The ED-L1 and L1-TR LMP1 promoters each contain STAT binding sites. (a) EMSA showing binding of purified, activated STAT1 and STAT4 to ^{32}P-labeled oligonucleotide probes containing the potential STAT binding sites from the Qp, ED-L1, and L1-TR promoters. The control STAT1 and STAT4 probes contain consensus STAT binding sites. Qp promotes EBNA1 expression in tumors. (b) Competition and supershift EMSAs illustrating the specificity of STAT4 binding to the L1-TR promoter probe. The L1-TR(mt) competitor contains a mutated STAT binding site. The Flag competitor oligonucleotide and antibody were used as controls for nonspecific effects.

(Fig. 3b). Transfection of L1-TRp–CAT into cell lines that were mutant for JAK1 or TYK2 resulted in decreased reporter expression compared to that observed in the parental cell line. In contrast, a control thymidine kinase promoter-CAT reporter was equally well expressed in mutant and parental cell lines. Mutation of the STAT site within the L1-TR promoter in L1-TRmtp–CAT led to significantly reduced CAT expression in the parental cell line, and there was a small additional loss of activity in the JAK1- and TYK2-negative cell lines.

The B95-8 lymphoblastoid cell line expresses LMP1 predominantly from the ED-L1 promoter, but low levels of L1-TR-initiated transcripts can be detected in B95-8 cells by RT-PCR analysis. B95-8 cells were subjected to RT-PCR analysis for L1-TR-initiated mRNAs before and after treatment with IL-6 (Fig. 4). Addition of IL-6 increased L1-TR activity, as indicated by the increased amount of the L1-TR-specific 749-bp RT-PCR product. The amount of control EBER RNA (EBV-encoded small nonpolyadenylated RNA) detected was not affected by IL-6 treatment. This set of experiments provides evidence that STATs not only bind to the L1-TR promoter but also positively regulate its activity.

Evidence for STAT3 as a specific regulator of the Qp and L1-TR promoters. We have provided evidence that EBV latency promoters can be activated by JAK-STAT signaling. In normal circumstances, activation of STATs is a transient response to cytokines or growth factors. However, certain oncogenes, including v-Src of Rous sarcoma virus, can cause constituitive STAT activation. The cellular homolog of v-Src, c-Src, is a member of a nonreceptor protein tyrosine kinase family that associates with the plasma membrane. In v-Src-transformed cells, JAK1 and to a lesser extent JAK2 kinases are constituitively activated, as are STAT3, STAT5, and STAT1 (7, 54, 61, 62). We wished to address whether EBV latency promoters would respond to aberrant oncogene-induced STAT activation. The effect of v-Src on reporter expression directed by the EBNA1 Qp and LMP1 ED-L1 and L1-TR promoters was examined in transfected HeLa cells (Fig. 5). Expression from each of these three latency promoters was significantly upregulated in the presence of v-Src. To evaluate the extent to which the promoter response was mediated by STAT3, v-Src was also transfected in the presence of STAT3β, a dominant negative inhibitor of STAT3. STAT3β is a natu-

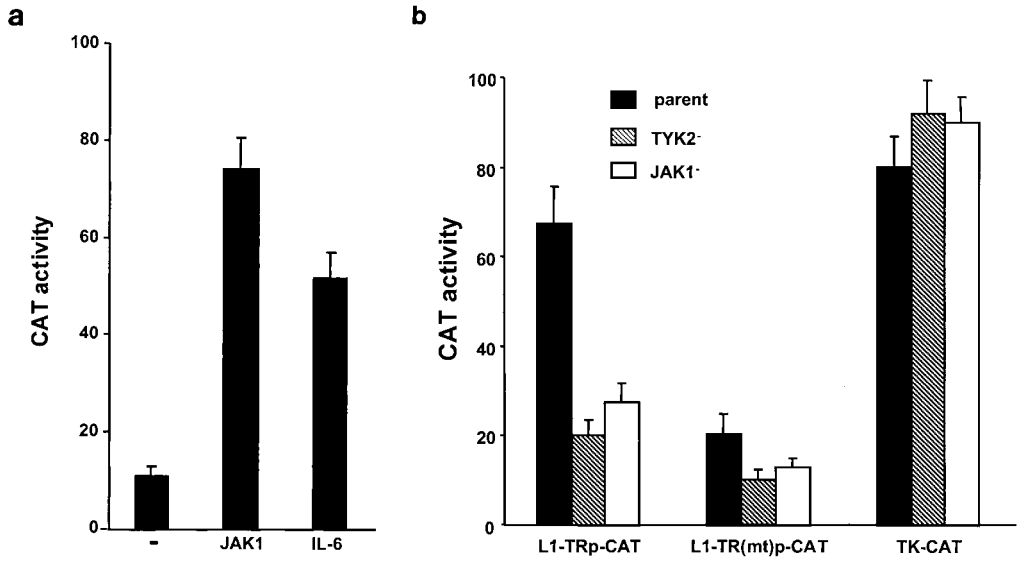

FIG. 3. The L1-TR LMP1 promoter is responsive to the STAT activators JAK1 and IL-6 and has reduced activity in JAK mutant cell lines. (a) Reporter assay showing induction of CAT expression from L1-TRp–CAT in HeLa cells cotransfected with a JAK1 expression vector or treated for 48 h with human IL-6 (100 ng/ml). The results shown are an average of three experiments, with the standard deviation indicated. (b) Reporter assay comparing CAT expression from L1-TRp–CAT and L1-TR(mt)p–CAT in parental (2fTGH) versus TYK2 (U1A) and JAK1 (U4A) mutant cells. The STAT binding site is mutated in L1-TR(mt)p–CAT. The promoter in TK-CAT is the non-STAT-regulated herpes simplex thymidine kinase promoter. The results shown are an average of three experiments, with the standard deviation indicated.

rally occurring splice variant of STAT3 that is deleted in the carboxy-terminal activation domain (6). The v-Src-induced activation of the EBNA1 Qp promoter was substantially inhibited by STAT3β, while the activation of the LMP1 L1-TR promoter was completely abolished. In contrast, the v-Src-induced activation of the ED-L1 LMP1 promoter was not affected by STAT3β, indicating that the two LMP1 promoters are responsive to different STATs. The data strongly implicate STAT3 as a biologically relevant STAT for the activation of the Qp and L1-TR promoters and raise the possibility that aberrant activation of STAT3 may be a contributing factor in EBV-associated pathogenesis.

NPC and Hodgkin's disease tumor cells contain activated, nuclear STATs. There is growing evidence for the presence of constituitively activated STATs in a variety of tumors. For example, constituitive activation of STAT3 has been observed in head and neck squamous cell carcinomas (23). We have provided evidence that the EBNA1 Qp and LMP1 promoters expressed in tumors are STAT responsive, with the biologically relevant STAT for Qp and the L1-TR LMP1 promoter likely to be STAT3 while the LMP1 ED-L1 promoter apparently responds to a different STAT, possibly STAT5 or STAT1. The issue arises as to the status of STATs in EBV-associated tumors. Immunohistochemistry was used to examine the intracellular localization of STAT3, STAT1, and STAT5 in archival samples of Hodgkin's disease and NPC tissues (Fig. 6 and 7). The EBV status of the Hodgkin's disease tissues was determined using standard EBER RNA in situ hybridization (data not shown). The malignant Reed-Sternberg cells in Hodgkin's disease showed both cytoplasmic and nuclear staining for STAT3. The presence of the activated, nuclear form of STAT3 was detected in both EBV-positive and EBV-negative Hodgkin's disease tissue samples (Fig. 6b and d). Immunohistochemistry performed for STAT1 on the same Hodgkin's disease tissues showed a low level of activated, nuclear STAT1 in this EBV-negative sample (Fig. 6c) but only cytoplasmic STAT1 staining in the EBV- positive tissue (Fig. 6a). The NPC tissue showed cytoplasmic STAT staining plus strong nuclear staining for both STAT3 and STAT1 (Fig. 6e and f and Fig. 7a and b) and STAT5 (Fig 7c). Interestingly, there was heterogeneity within the NPC tissue, with a mixture of strongly staining positive nuclei and adjacent negative nuclei. Whether this represents true heterogeneity of STAT activation in individual cells or has a technical basis is not currently clear.

STAT proteins are not indiscriminately activated in NPC. Tissue was also stained for STAT4. Cytoplasmic signal was

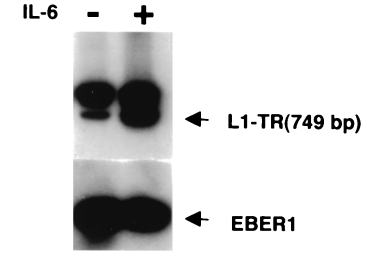

FIG. 4. The endogenous L1-TR promoter in B95-8 cells is activated by IL-6. Southern blot analyses of RT-PCR products generated from B95-8 lymphoblastoid cells using the P1 and P2 primers for L1-TRp initiated mRNA (top) or primers for the polymerase III EBER1 RNAs (bottom) are shown. cDNAs were detected using specific ^{32}P-labeled oligonucleotide probes. Growth of B95-8 cells in medium containing IL-6 increased the amount of the 749-bp L1-TR-initiated LMP1 mRNA but did not affect EBER1 RNA levels. The larger PCR product was also generated in the absence of the RT reaction.

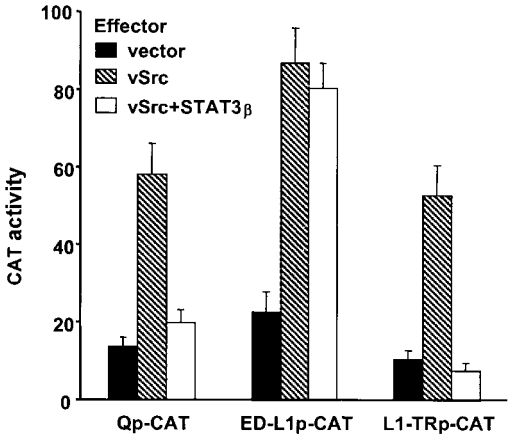

FIG. 5. The EBNA1 Qp and both LMP1 promoters are activated by v-Src-induced signaling and differentially repressed by interference with STAT3 function. Reporter assay in HeLa cells cotransfected with the EBV latency Qp-CAT, ED-L1–CAT, and L1-TR–CAT constructions and either control vector DNA (vector), an expression plasmid for v-Src, or v-Src plus a dominant negative STAT3 inhibitor (STAT3β). The results shown are an average of three experiments, with the standard deviation indicated.

observed, but no evidence for nuclear, activated STAT4 was detected (Fig. 7d). A comparison of the roles of EBNA2 and STATs in regulating viral and cellular gene expression in EBV-associated tumors is presented in Fig. 8.

DISCUSSION

In EBV-associated tumors, viral expression is restricted to a limited number of the latency genes. EBNA1 is always expressed, and LMP1 is frequently expressed. We have previously shown that the Qp promoter that drives EBNA1 expression in EBV-associated tumors is JAK-STAT regulated, and we now present evidence that this pathway also controls expression of the oncogenic LMP1 protein. The well-characterized ED-L1 promoter drives LMP1 synthesis in B cells, but a second promoter located upstream of ED-L1 and inside the terminal repeat region of the L1-TR genome also directs LMP1 transcription in NPC (45, 53). We have now detected L1-TR-initiated transcripts in an EBV-positive B-cell line treated with IL-6 and in a sample of EBV-positive Hodgkin's disease tissue, and this suggests that usage of this promoter is not restricted to epithelial tumors. Mobility shift assays demonstrated functional STAT binding sites in the ED-L1 and L1-TR promoters. Furthermore, reporter assays showed that both LMP1 promoters responded to activation of the JAK-STAT pathway, whether it be by the addition of the cytokine IL-6 or by cotransfection of JAK1 or the oncogene v-Src.

Seven independent STAT proteins have been identified to date. Studies utilizing modified STATs that are constitutively activated have implicated STAT5 and STAT3 as the STATs whose properties suggest an ability to contribute to cell transformation by promoting cell cycle progression or preventing apoptosis (4). The antiapoptotic protein Bcl-x_L and the *mcl-1* gene are induced by activated STAT5 and STAT3 (10, 26). Both STAT3 and STAT5 also increase the expression of cyclin D1, which controls cell cycle progression from G_1 to S phase (5, 37). c-Myc, a transcription factor that affects both cellular proliferation and cell survival, is upregulated by STAT3 (33). On the other hand, although activation of STAT1 has been described in conjunction with either STAT5 or STAT3 in a variety of tumors, constituitive activation of STAT1 alone appears to mediate growth-inhibitory effects.

Although STATs recognize similar binding sites in vitro, promoter responses are STAT specific in vivo. The results of transfection experiments using v-Src and the dominant negative STAT3β strongly suggest that STAT3 is the biologically relevant STAT for activation of Qp and the L1-TR LMP1 promoter. Both promoters were upregulated by cotransfection of v-Src, and this stimulation was abolished by STAT3β. Interestingly, the standard LMP1 promoter response to v-Src was not affected by STAT3β, suggesting that one of the other STATs activated by v-Src, either STAT5 or STAT1, is responsible for the STAT-mediated responses of this promoter. The presence of two LMP1 promoters that respond to different STATs may expand the circumstances in which LMP1 can be expressed. For example, the malignant Reed-Sternberg and Hodgkin cells in EBV-positive Hodgkin's disease tumors express particularly high levels of LMP1 and, in our immunohistochemical analyses, contained nuclear, activated STAT3 but

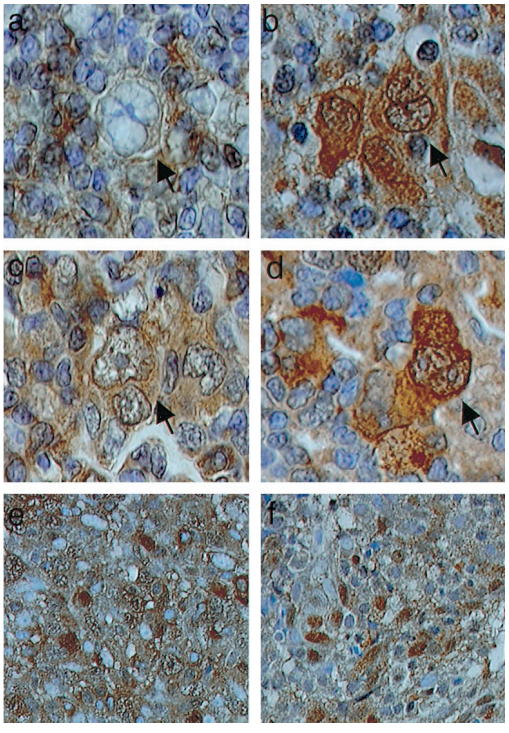

FIG. 6. NPC and Hodgkin's disease Reed-Sternberg cells contain activated nuclear STATs. Immunohistochemical analyses of STAT localization in NPC and Hodgkin's disease tissue samples are shown. (b and d) STAT3 staining in EBV-positive (b) and EBV-negative (d) Hodgkin's disease tissue. The malignant Reed-Sternberg cells are indicated by arrowheads. (a and c) STAT1 staining in EBV-positive (a) and EBV-negative (c) Hodgkin's disease tissue. (e and f) NPC tissue stained for STAT1 (e) and STAT3 (f). STATs were detected using anti-STAT1 and anti-STAT3 primary antibodies (Santa Cruz), and reactive complexes were visualized using StrpABComplex/horseradish peroxidase (Dako). Tissue was counterstained with hematoxylin.

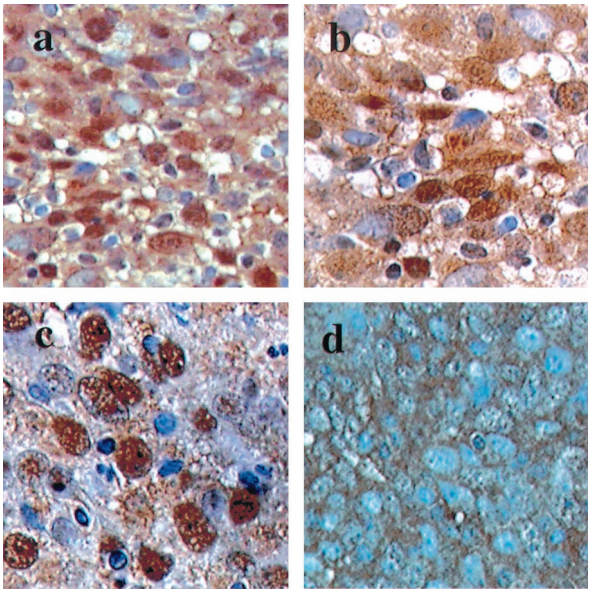

FIG. 7. Further evaluation of the intracellular localization of STATs in NPC tissues. Immunohistochemical staining was performed as described in the legend to Fig. 6. STAT3 and STAT5 staining is visible within tumor cell nuclei. In contrast, STAT4 staining is restricted to the cytoplasm. (a) STAT3 (magnification, ×400). (b) STAT3 (magnification, ×600). (c) STAT5 (magnification, ×600). (d) STAT4 (magnification, ×600).

not STAT1. We detected LMP1 expression from the L1-TR promoter in Hodgkin's disease tissue, and this would be consistent with STAT3 regulation of this promoter. On the other hand, we and others (45) found evidence for both ED-L1- and L1-TR-initiated LMP1 transcripts in NPC tissues, and, in contrast to the results obtained with Hodgkin's disease, we also detected nuclear STAT5 and STAT1 in NPC tissue.

Promoter responsiveness to STATs is also modified by interactions with other transcription factors. In this regard, it is interesting that SP1 and SP3 contribute to the basal activity of the L1-TR promoter (45, 53). Transcriptional cooperativity between Sp1 and STAT3 has been described for IL-6-mediated activation of the C/EBPδ promoter (8), and Sp1 and STAT1 also directly interact to produce cooperative transcriptional responses (36, 63).

The observation that the EBNA1 and LMP1 genes that are expressed in EBV-associated tumors are STAT regulated, along with the recognition that STATs are frequently aberrantly activated in human cancers, including those that are EBV associated, provides a background for speculating on the etiology of EBV-associated tumorigenesis. Long-term EBV latency is established in memory B cells. Only LMP2A and the *Bam*HI-A rightward transcripts are constitutively expressed in these cells (13, 39). Long-lived memory B cells originate in germinal centers, where cells are selected in part on the basis of competition for antigen. In transgenic mice expressing Bcl-2, the antiapoptotic function of Bcl-2 allows survival in the memory compartment of B cells that lack affinity-enhancing somatic gene mutations (48). LMP1 plays an antiapoptotic role that includes upregulation of cellular Bcl-2 expression and is mediated through NF-κB induction (28). We have argued that LMP1 expression is STAT regulated. Primary B lymphocytes do not express activated STATs (31, 58), but antigen receptor engagement in B cells induces nuclear expression of STAT5 and STAT6 and interactions between B cells and follicular

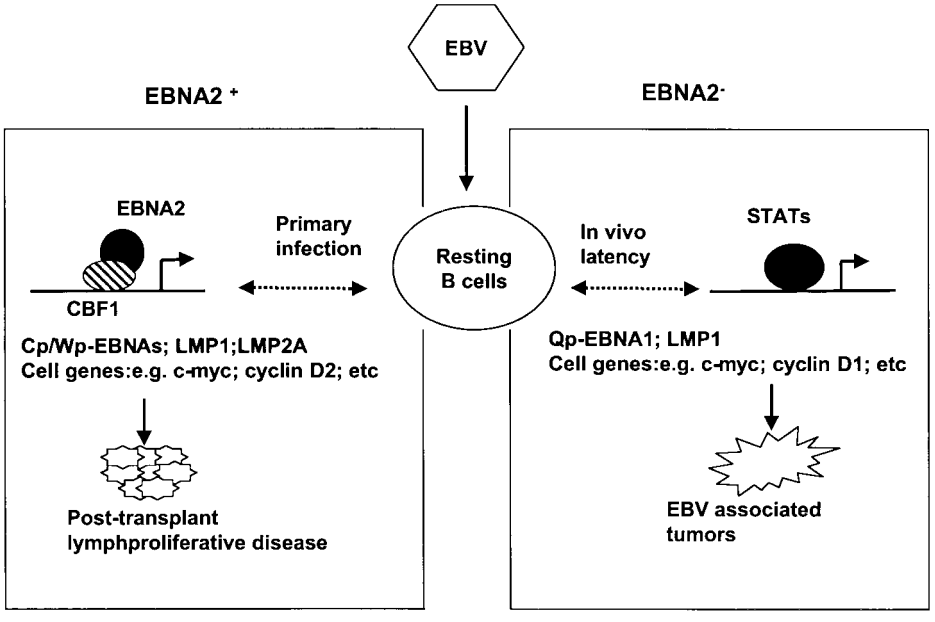

FIG. 8. Model for in vivo EBV gene regulation and tumorigenesis. In primary infection, EBNA2 regulates the expression of the nuclear EBNAs and the LMP genes including LMP1 and modulates cellular gene expression. The strong immune response to the immunogeneic EBNAs limits the occurrence of EBNA2-expressing tumors to immunocompromised individuals. During in vivo latency, in the absence of EBNA2, the EBNA1 and LMP1 genes are regulated by STATs. Chronic activation of STATs through a natural cytokine signaling event such as inflammation or through aberrant oncogene-activated signaling may upregulate EBV EBNA1 and LMP1 expression and predispose the cell to EBV-driven tumorigenesis.

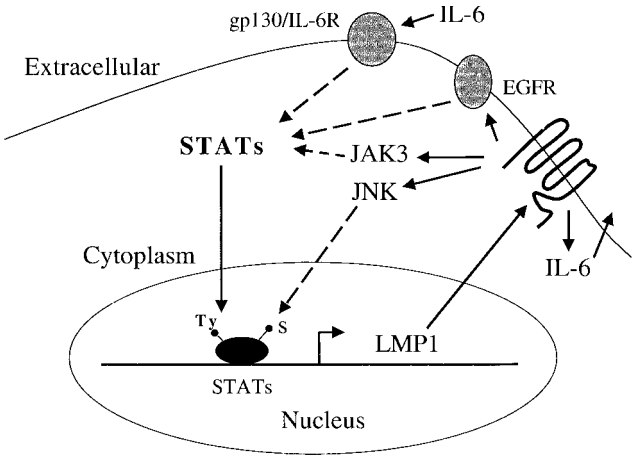

FIG. 9. Potentiation of STAT signaling by LMP1. LMP1 may contribute to a self-sustaining cycle of STAT activation and continued LMP1 synthesis. LMP1 upregulates expression of the cytokine IL-6 and EGFR, which mediate tyrosine phosphorylation of STAT1 and STAT3. LMP1 is also able to activate JAK3, whose targets include STAT5. In addition, LMP1 increases the activity of JNK, a kinase involved in serine phosphorylation of the STAT protein transcriptional activation domain. Thus, STAT-induced LMP1 expression may lead to a state of constitutive STAT activation that can be maintained independently of ongoing external signaling.

dendritic cells and T cells in the germinal center elicit cytokine responses that also involve STATs (16, 31, 52). Transient stimulation of LMP1 expression in the germinal center could provide an additional survival signal that would allow EBV-carrying memory cells to transit the germinal center without undergoing apoptosis. Further, the expression of EBNA1 would ensure maintainance of the EBV genome in B cells undergoing transient proliferation in the germinal center.

The sporadic nature of EBV-associated tumorigenesis in the face of ubiquitous, lifelong infection has always posed a conundrum that can only partially be explained in terms of host immune regulation. The natural STAT responsiveness of the Qp EBNA1 and LMP1 promoters may be one of the factors in this sporadic pathogenesis. It has proven difficult to detect EBV infection in normal mucosal epithelium despite the fact that virus is continuously shed into the saliva of EBV-seropositive individuals. Activated STATs are not usually present in normal epithelium, but nuclear STAT3 has been detected in the normal mucosa of patients with head and neck cancers (23). Entry of EBV into a cell that has already undergone disregulation of STAT signaling could be a predisposing event for EBV-associated tumorigenesis. Along similar lines, we observed nuclear STAT3 in both EBV-negative and EBV-positive samples of Hodgkin's disease tissue, suggesting that aberrant STAT3 activity may be a common feature of the development of this malignancy and may precede EBV infection. However, there does also exist the possibility for establishment of a positive autoregulatory loop of LMP1 expression and STAT activation (Fig. 9). LMP1 signaling induces the expression of IL-6 and the epidermal growth factor receptor (EGFR) (18, 20, 25, 38) both of which activate STATs, including STAT3. Upregulation of EGFR expression is seen in squamous cell carcinoma of the head and neck, and it has been proposed that EGFR overexpression is an early event in the development of these tumors (49). LMP1 is also able to activate JAK3, whose targets include STAT5 (22). Further, LMP1 induces the expression of JNK, one of the kinases that facilitate STAT activity through serine phosphorylation of the STAT transcriptional activation domain. Thus, prolonged activation of normal JAK-STAT signaling through, for example, chronic inflammation may, in some circumstances, be sufficient to establish a pattern of EBV gene expression that is potentially both self-sustaining and tumorigenic.

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