BET proteins promote efficient murine leukemia virus integration at transcription start sites

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The selection of chromosomal targets for retroviral integration varies markedly, tracking with the genus of the retrovirus, suggestive of targeting by binding to cellular factors. γ-Retroviral murine leukemia virus (MLV) DNA integration into the host genome is favored at transcription start sites, but the underlying mechanism for this preference is unknown. Here, we have identified bromodomain and extraterminal domain (BET) proteins (Brd2, -3, -4) as cellular-binding partners of MLV integrase. We show that purified recombinant Brd4(1-720) binds with high affinity to MLV integrase and stimulates correct concerted integration in vitro. JQ-1, a small molecule that selectively inhibits interactions of BET proteins with modified histone sites impaired MLV but not HIV-1 integration in infected cells. Comparison of the distribution of BET protein-binding sites analyzed using ChIP-Seq data and MLV-integration sites revealed significant positive correlations. Antagonism of BET proteins, via JQ-1 treatment or RNA interference, reduced MLV-integration frequencies at transcription start sites. These findings elucidate the importance of BET proteins for MLV integration efficiency and targeting and provide a route to developing safer MLV-based vectors for human gene therapy.

retroviral gene therapy | virus–host interactions

Integrating retroelements often use cellular-binding partners to direct integration of reverse-transcribed DNA into chromatin. For yeast retrotransposons, cellular-binding partners have been identified and shown to target integration in vivo for several Ty element integrase (IN) enzymes (1–4). The selection of chromosomal targets for retroviral integration varies markedly, tracking with the genus of the retrovirus studied (5–7). For example, the γ-retroviruses favor integration near transcription start sites, whereas lentiviruses favor integration within transcription units. These observations have suggested that different cellular-binding partners of retroviral integrases are likely to be responsible for integration target-site selection. However, to date, only one example has been reported: lens epithelium-derived growth factor (LEDGF/p75), which functions as a bimodal tether that engages HIV-1 intasomes and navigates them to active genes (8–14). Cellular cofactors of other retroviral genera are currently unknown.

The molecular mechanisms of γ-retroviral murine leukemia virus (MLV) integration are of particular significance because MLV-based vectors are used for human gene therapy. In clinical trials, the use of γ-retroviral vectors to correct primary immunodeficiencies has been curative, but adverse events have occurred associated with insertion of MLV-based vectors near protooncogenes (reviewed in refs. 15–18). The identification of cellular factors for γ-retroviruses may provide mechanistic clues to facilitate the development of safer gene-therapy vectors.

In this report, we have identified the bromodomain and extraterminal domain (BET) proteins (Brd2, -3, -4) as the cellular-binding partners of MLV IN and demonstrate their significance for stimulating and targeting MLV integration at transcription start sites.

Results

BET Proteins Specifically Interact with MLV IN. To identify cellular-binding partners of MLV IN, we used affinity capture coupled with mass spectrometry (MS). In parallel experiments, the interacting partners of MLV and HIV-1 INs from nuclear extracts of NIH 3T3 and Sup-T1 cells were compared. The semiquantitative analyses of MS peaks identified the BET proteins (Brd2, -3, and -4) as the main binding partners of MLV IN (Table 1, Table S1, and Fig. S1). Of these, Brd4 and Brd3 were the top hits in NIH 3T3 and Sup-T1 cells, respectively. Differential pull-down levels of these proteins (Table 1) could be attributable to the varying expression levels of BET proteins seen in different cell types (Fig. S2). In control experiments, no peptides from the BET proteins were detected in HIV-1 IN pull-downs. Instead, as expected (8), LEDGF/p75 was identified as the main interacting partner of HIV-1 but not MLV IN. Furthermore, the immunoblot analyses of the pull-down fractions (Fig. 1 *A* and *B*) have validated our MS results by showing that all three BET proteins selectively bind MLV IN but not HIV-1 IN. The interaction between MLV IN and Brd2 has also been detected by yeast two-hybrid experiments (19).

We next attempted to map the interacting domains between MLV IN and full-length Brd3(1-726). MLV IN is composed of the following three distinct domains (Fig. S3*A*): the N-terminal domain (NTD), which like prototype foamy virus IN (20) is comprised of the NTD-extension domain and the HH-CC–type Zn finger; the catalytic core domain (CCD) containing the DDE triad that coordinates catalytic Mg^{2+}; and the C-terminal domain (CTD), which is thought to bind DNA, but it could also have additional functions. Similar levels of ectopically expressed Brd3(1-726) were pulled down by full-length MLV IN and its CTD (Fig. 1*C* and Fig. S3*B*). In contrast, no interactions were detected between Brd3 (1-726) and the MLV IN NTD or the two domain (NTD+CCD) construct. These findings indicate that MLV IN CTD is primarily responsible and sufficient for interaction with Brd3(1-726).

To delineate the Brd3(1-726) segments (Fig. S4) interacting with MLV IN, two truncated constructs were studied: Brd3 (1-419), the N-terminal fragment which contained the two bromodomains known to interact with chromatin (21–25); and Brd3 (420-726), the C-terminal region implicated in a number of protein-protein interactions (reviewed in refs. 26–28). Full-length

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Table 1. List of top protein hits from NIH 3T3 and Sup-T1 cells specifically binding GST-MLV IN or GST–HIV-1 IN

	Cells			
	NIH 3T3		Sup-T1	
Protein	MLV IN	HIV-1 IN	MLV IN	HIV-1 IN
Brd4	164	ND	18	ND
Brd2	90	ND	7	ND
Brd3	88	ND	25	ND
LEDGF/p75	ND	35	ND	15

"Unweighted spectrum count" values for each protein are shown from representative runs of four independent experiments. "ND" indicates that the listed protein was not detected.

Brd3(1-726) (Fig. 1*D*) and its C-terminal Brd3(420-726) (Fig. 1*E*) bound MLV IN, whereas the N-terminal Brd3(1-419) failed to bind MLV IN (Fig. 1*F*). Similar results were seen with recombinant MLV IN binding to recombinant Brd4(1-720) and Brd4 (462-720) but not Brd4(1-461) (Fig. S5).

BET Proteins Directly Bind and Stimulate MLV Integration in Vitro. To examine whether the observed interactions were direct or bridged by additional cellular components, we next examined interactions with purified recombinant proteins. Among the three BET proteins, only His-Brd4(1-720) was amenable to bacterial expression and purification. Importantly, Brd4(1-720) contains all of the functional domains and motifs conserved between Brd2, -3, and -4 (Fig. S4) and, thus, serves as a representative of all three BET proteins. The data in Fig. 2*A* show that Brd4(1-720) binds MLV but not HIV-1 IN. In control experiments, LEDGF/p75 interacted with HIV-1 but not MLV IN (Fig. 2*B*). Using pull-down experiments, the apparent K_d value was ~33 nM for MLV IN-Brd4(1-720) (Fig. 2 *C* and *D*). A K_d of ~200 nM was measured for the HIV-1 IN-LEDGF/p75 interaction (29).

We next examined the effects of Brd4(1-720) in in vitro-integration assays catalyzed by MLV IN (Fig. 2 *E* and *F*). Addition of Brd4(1-720) to the reactions significantly enhanced the biologically relevant concerted two-end integration products. Comparative analysis of strand-transfer activities of MLV and HIV-1 INs revealed that the levels of stimulation for the integration activities of MLV IN by Brd4(1-720) and HIV-1 IN by LEDGF/p75 were comparable [Fig. 2 *F* and *G*; ~564% for MLV IN+Brd4(1-720) and ~689% for HIV-1 IN+LEDGF/p75]. In control experiments, no stimulation of strand transfer activities of MLV IN by LEDGF/p75 or HIV-1 IN by Brd4(1-720) were

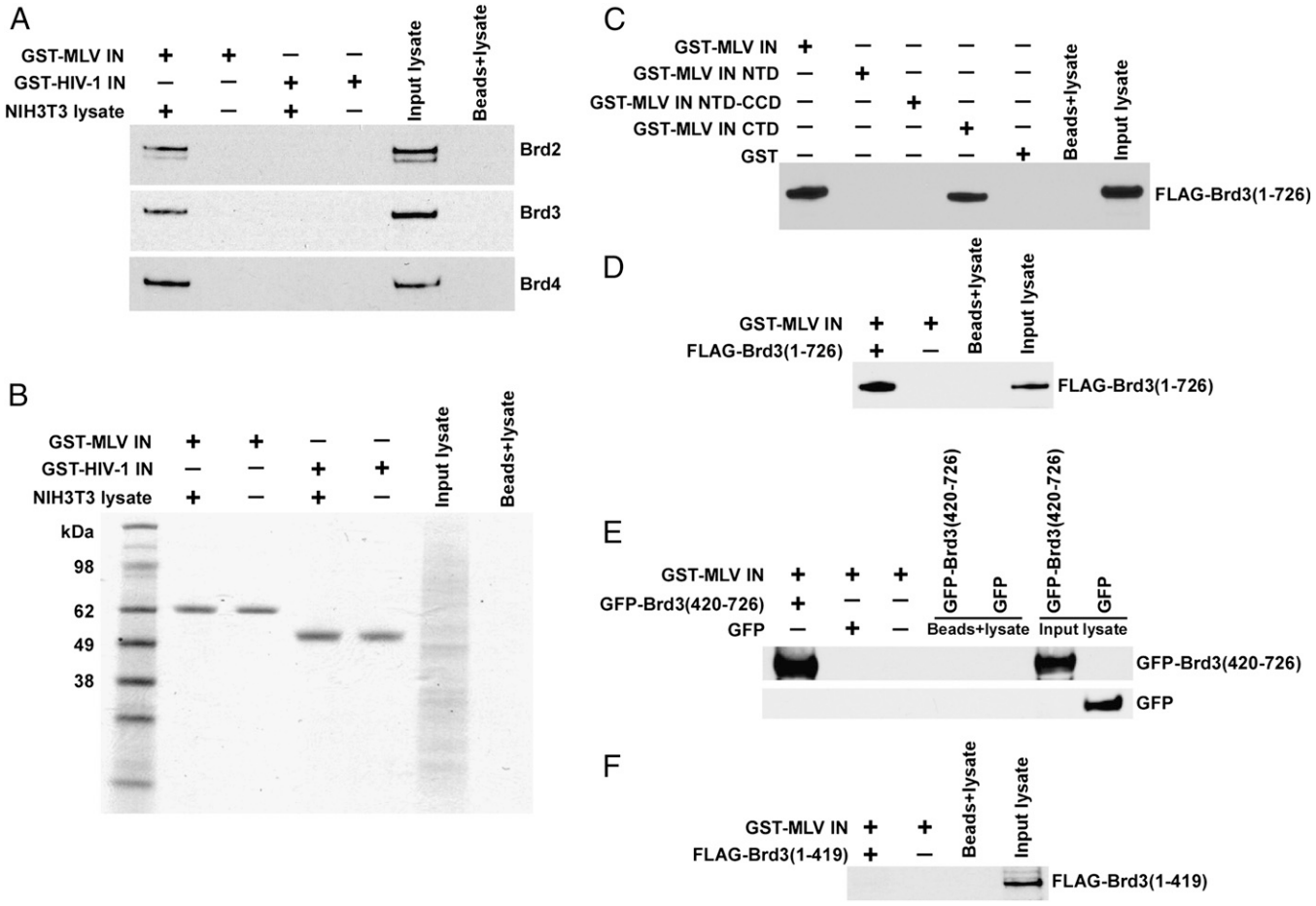

Fig. 1. BET proteins specifically interact with MLV integrase. (*A*) GST pull-down of NIH 3T3 cell lysate with either GST-MLV IN or GST–HIV-1 IN and immunoblotting with Brd2, -3, and -4 antibodies. (*B*) Coomassie-stained SDS/PAGE gel of GST pull-down products from *A* showing that similar levels of GST-MLV IN or GST–HIV-1 IN bound to glutathione Sepharose beads. (*C*) GST pull-down of HEK293T cell lysate expressing FLAG-Brd3(1-726) with GST-MLV IN, GST-MLV IN CTD, GST-MLV IN NTD, and GST-MLV IN NTD-CCD and immunoblotting with FLAG antibody. (*D–F*) Affinity pull-down with GST-MLV IN and HEK293T cell lysate expressing FLAG-Brd3(1-726) (*D*), GFP or GFP-Brd3(420-726) (*E*), and FLAG-Brd3(1-419) (*F*), respectively. Immunoblotting with FLAG or GFP antibody. "Input lysate (10%)" indicates 10% of indicated whole-cell lysate used for pull-down. "Beads + lysate" indicates control pull-down without GST-tagged protein.

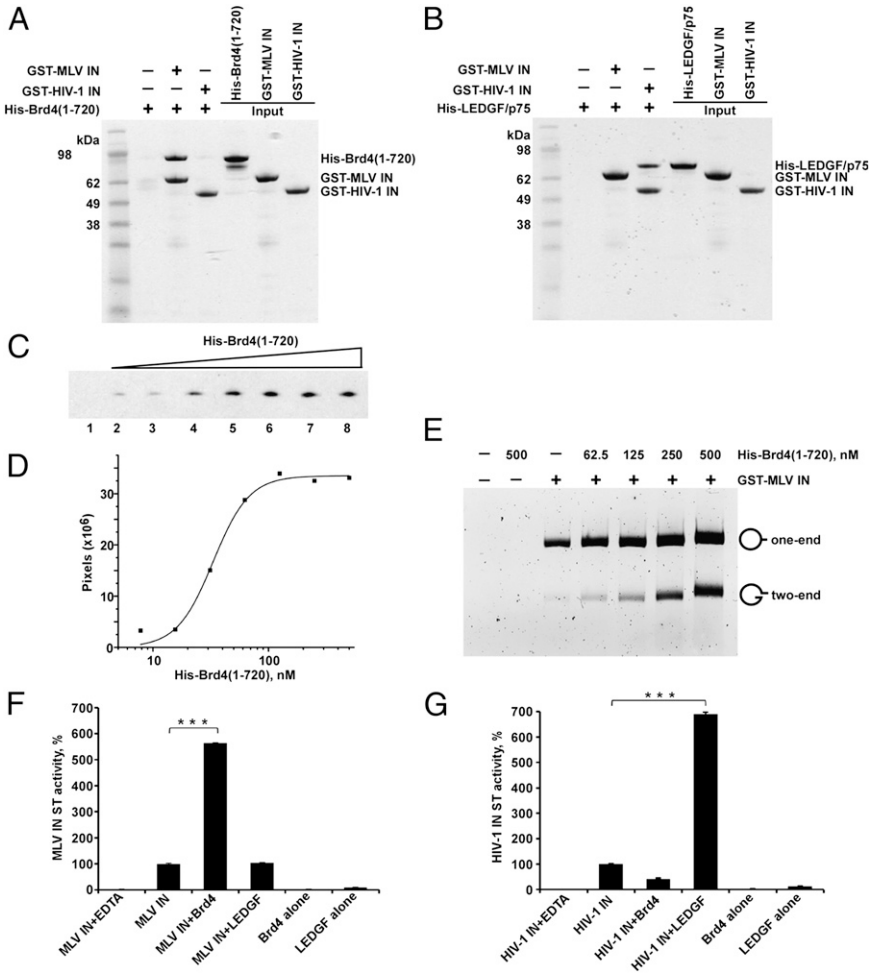

Fig. 2. BET proteins directly bind and stimulate MLV integrase activity in vitro. (*A*) GST pull-down of purified recombinant His-Brd4(1-720) (5 μM) with either GST-MLV IN or GST–HIV-1 IN. GST-MLV IN and GST–HIV-1 IN inputs indicate control pull-down without His-Brd4(1-720). Also shown is the 10% of His-Brd4(1-720) as input. (*B*) GST pull-down of purified recombinant His-LEDGF/p75 (5 μM) with either GST-MLV IN or GST–HIV-1 IN. GST-MLV IN and GST–HIV-1 IN inputs indicate control pull-down without His-LEDGF/p75. Also shown is the 10% of His-LEDGF/p75 as input. (*C*) Affinity pull-down of increasing concentrations (lanes 2–8) of purified recombinant His-Brd4(1-720) with GST-MLV IN. Control pull-down is shown with 125 nM His-Brd4 (1-720) without GST-MLV IN (lane 1) to rule out nonspecific binding to glutathione beads. (*D*) Graphical representation of immunoblot shown in *C* to determine the apparent K_d of binding of ∼33 nM. The intensities of GST-MLV IN-bound His-Brd4(1-720) bands were quantified using ImageJ software, and data were fit to the Hill equation. (*E*) Concerted integration of 5′-Cyanine 5 (Cy5)–labeled viral donor DNA (1 μM) into target DNA (pBR322; 300 ng) by purified recombinant His-MLV IN (0.3 μM). Purified recombinant His-Brd4(1-720) was added to the reactions at the indicated concentrations. The image represents Cy5 signal as detected by Typhoon 9410 Imager. Indicated are the "one-end" and the biologically relevant concerted "two-end" integration products. (*F* and *G*) Effects of Brd4(1-720) and LEDGF/p75 on in vitro strand transfer (ST) activity of MLV IN (*F*) and HIV-1 IN (*G*). The ST products were detected by measuring the HTRF signal. Recorded signals were normalized using 100% ST activity for integrase alone. Bars represent means ± SD [$n = 3$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student t test for IN alone vs. indicated MLV IN+Brd4(1-720) or HIV-1 IN+LEDGF/p75 samples, respectively].

observed (Fig. 2 *F* and *G*). Thus, BET proteins and LEDGF/p75 specifically stimulate MLV and HIV-1 integration, respectively.

Antagonism of BET Proteins Reduces MLV Integration in Infected Cells. To elucidate the role of the BET proteins in MLV replication, we down-regulated these cellular proteins using either siRNA or shRNA. The individual down-regulation of BET proteins using a siRNA approach impaired MLV but not HIV-1 expression by ∼23% for Brd2, ∼32% for Brd3, and ∼27% for Brd4 (Fig. S6 *A–C*). Concurrent down-regulation of all three BET proteins by siRNA impaired MLV but not HIV-1 expression by ∼37% (Fig. S6 *D–F*). In contrast, as reported previously (30, 31), HIV-1 expression was enhanced upon Brd4 down-regulation (Fig. S6 *C* and *F*). More effective down-regulation of individual BET proteins was obtained with shRNA, which impaired MLV but not HIV-1 expression by ∼39% for Brd2, ∼37% for Brd3, and ∼49% for Brd4 (Fig. S7 *A* and *B*). Our results in Fig. 1 suggest that Brd2, -3, and -4 are redundant in interaction with MLV IN. Attempts to knock down all three proteins concurrently using shRNA were lethal to the cells.

Therefore, we used the cell-permeable small molecule JQ-1, which selectively inhibits interactions of all three BET proteins with cognate-modified histone sites (32). JQ-1 inhibited MLV expression in a dose-dependent manner (Fig. 3*A*). In contrast, as expected (30, 31), HIV-1 expression was enhanced in JQ-1–treated cells. Thus, our results indicate that the specific inhibition of BET proteins selectively impairs MLV expression.

To pinpoint the replication steps affected by JQ-1 treatment, we quantified viral DNA forms longitudinally, including the minus-strand strong-stop extension products (MSSEs), plus-strand extension products (PSEs), 2-LTR circles, and integrated proviruses (33). JQ-1 treatment did not alter MSSEs or PSEs (Fig. 3 *B* and *C*), indicating that MLV reverse transcription was not affected. In contrast, a significant increase in 2-LTR circles was observed upon JQ-1 treatment (Fig. 3*D*). These dead-end products serve as surrogate markers and are known to increase in quantity in the presence of defective integration (33). Quantitation of integrated proviruses by Alu-based quantitative (q) PCR revealed a significant reduction in integrated MLV proviruses upon treatment with JQ-1 (Fig. 3*E*). In contrast, JQ-1 treatment had no significant effects on HIV-1 late reverse-transcription products (Fig. 3*F*), 2-LTR circles (Fig. 3*G*), or integrated proviruses (Fig. 3*H*). Additionally, we evaluated the effects of JQ-1 on the integration of a MLV-based retroviral vector. JQ-1 treatment significantly inhibited integration in a dose-dependent manner, which correlated closely with the levels of reduction of MLV vector expression (Fig. 3 *I* and *J*). Taken together, these results indicate that the inhibition of the BET proteins by JQ-1 selectively impaired MLV integration and, hence, subsequent gene expression.

BET Proteins Target MLV Integration to Transcription Start Sites. We next evaluated whether there is a correlation between MLV-integration sites and the chromatin-binding sites of BET proteins mapped using ChIP-Seq data (21). For this, MLV-integration sites in HEK293T cells were compared within a 1-kb window to Brd2, -3, and -4 binding sites (21). Fig. 4*A* shows that compared with HIV-1 or avian sarcoma leukosis virus (ASLV), MLV significantly favored integration near-binding sites of the BET proteins.

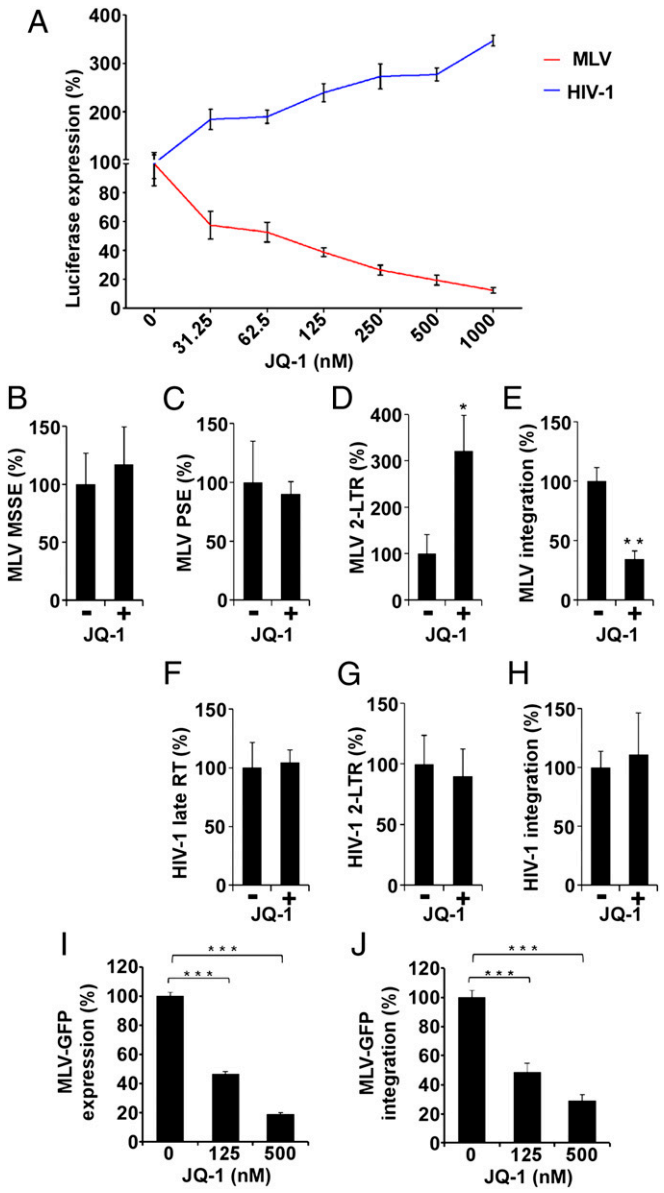

Fig. 3. Inhibition of BET proteins reduces MLV integration. (*A*) Dose-dependent effect of JQ-1 on HIV-1 or MLV expression. HEK293T cells infected with luciferase reporter HIV-1 (HIV-1–Luc) or transduced with MLV-Luc vector in the absence or presence of indicated concentrations of JQ-1 inhibitor. Luciferase assay was performed at 48 h postinfection or posttransduction. The luciferase signal obtained at 0 nM JQ-1 (DMSO alone) was set to 100% (values represent mean ± SD; $n = 3$). (*B–E*) qPCR analysis of JQ-1–treated (indicated as "+"; 1,000 nM) or nontreated (indicated as "–"; DMSO) HEK293T cells infected with vesicular stomatitis virus-glycoprotein G (VSV-G) pseudotyped MLV. Bar graphs indicate the amount of PCR products relative to nontreated sample at 24 h postinfection for MSSS (*B*), PSE (*C*), and 2-LTR circle (*D*) products. (*E*) Bar graph indicates the integrated provirus relative to nontreated sample at 10 d postinfection. (*F–H*) qPCR analysis of JQ-1 treated (indicated as "+"; 1,000 nM) or nontreated (indicated as "–"; DMSO) HEK293T cells infected with VSV-G pseudotyped HIV-1–Luc. Bar graphs indicate the amount of PCR products relative to nontreated sample at 24 h postinfection for late reverse-transcription (Late RT) (*F*) and 2-LTR circle (*G*) products. (*H*) Bar graph indicates the integrated provirus relative to nontreated sample at 10 d postinfection. All bars represent means ± SD ($n = 3$; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ by Student *t* test). (*I* and *J*) HEK293T cells transduced with MLV-LTR GFP vector in the absence or presence of indicated concentrations of JQ-1 inhibitor. (*I*) Dose-dependent effect of JQ-1 on expression of MLV-LTR GFP vector. The percentages of GFP-positive cells were quantified by FACS analysis at 48 h posttransduction. The number of GFP-positive cells for 0 nM JQ-1 (DMSO alone) was set to 100%. (*J*) Dose-dependent effect of JQ-1 on integration of MLV-LTR GFP vector. Genomic DNA was harvested 15 d posttransduction, and the integrated copies of MLV-LTR GFP vector were measured. Bar graph indicates the integrated vector relative to 0 nM JQ-1 (DMSO alone) sample. All bars represent means ± SD ($n = 3$; $^{***}P < 0.0001$ measured by one-way ANOVA; multiple comparisons of the JQ-1 treatment to the DMSO control used Dunnett simultaneous test).

In contrast, MLV did not favor integration near-binding sites for heterochromatin protein 1 (HP1α and HP1β), which were mapped in the same study, are known to be enriched in heterochromatin and serve here as controls. We next examined MLV-integration sites near the promoters (within 1-kb window) bound by the BET proteins. The heatmap in Fig. 4*B* shows that in comparison with HIV-1 or ASLV, MLV significantly favors integration near promoters associated with the BET proteins. In contrast, MLV did not favor integration near promoters bound by HP1α and HP1β.

To dissect the role of BET proteins in MLV integration-site selection, we analyzed the distribution of 11,968 unique integration sites in cells treated with JQ-1 or a pool of Brd2, -3, and -4 siRNAs [Brd(2+3+4)i] by 454 pyrosequencing. As expected (6, 34) in control experiments with either no inhibitor or scrambled siRNA (Sci) MLV integration was favored (39% of integration events) within 2-kb distance from RefSeq transcription start sites (Fig. 4 *C–F*). The JQ-1 treatment significantly reduced the frequency of MLV integration at transcription start sites in a dose-dependent manner (Fig. 4 *C* and *D*). Moreover, concurrent down-regulation of all three BET proteins by siRNA also significantly reduced the frequency of MLV integration at transcription start sites (Fig. 4 *E* and *F*). The residual MLV integration at transcription start sites observed with JQ-1 or siRNA treatments is likely attributable to suboptimal inhibition or down-regulation of BET proteins, respectively. Additionally, other cellular or viral factors may also contribute to residual targeting. Taken together, these results indicate that BET proteins target MLV integration to transcription start sites.

Discussion

The distributions of integrated proviruses in host genomes are not random and appear to be genus-specific. For example, γ-retroviruses such as MLV exhibit strong bias for integrating in the vicinity of transcription start sites and CpG islands (6), whereas lentiviruses, including HIV-1, prefer to integrate into active genes (12, 35). Previous studies (7) with chimeric viruses have shown that IN is the principal viral determinant for integration-site selectivity. The distribution of integration sites for a chimeric HIV-1 virus with the IN coding region replaced with its MLV counterpart was markedly different from wild-type HIV-1 but closely resembled MLV (7).

The mechanism of integration-site targeting is best understood for HIV-1. The site selectivity of HIV-1 integration is controlled by cellular protein LEDGF/p75 (9, 11, 12), which functions as a bimodal tether. The LEDGF/p75 C-terminal region directly engages lentiviral INs, whereas its N-terminal region containing the PWWP domain recognizes trimethylated H3 tails in chromatin (36–38) and, accordingly, directs HIV-1 integration into actively transcribed genes (9–12).

Our present studies reveal that the BET protein-mediated interaction links MLV IN to transcriptional start sites in chromatin. In particular, our data suggest that the BET proteins act as bimodal tethers (Fig. S8), with the C-terminal fragment directly interacting with MLV IN (Fig. 1) and the N-terminal bromodomains binding to acetylated H3 and H4 tails (32), which are found at the transcription start sites and strongly correlate with MLV-integration sites (34) in genomic DNA. These findings support the notion that INs from different retroviral genera

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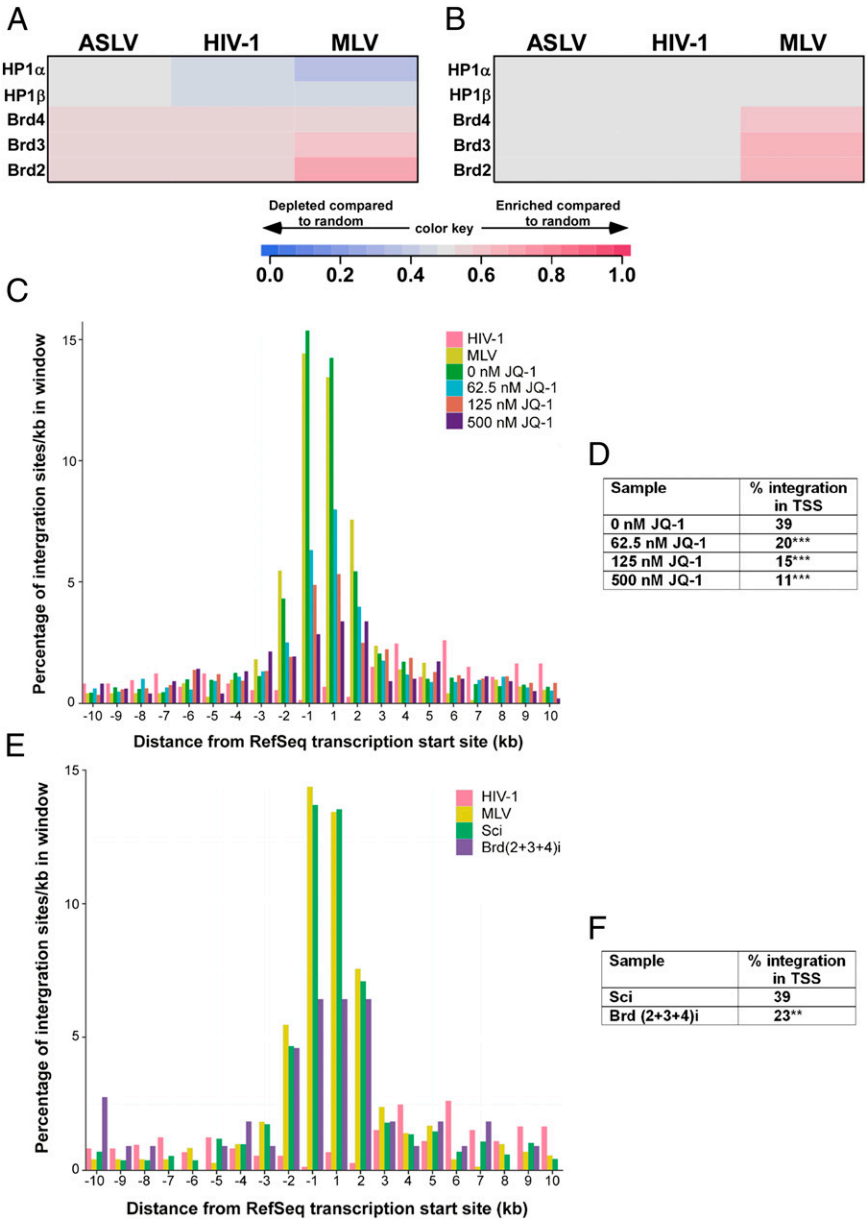

Fig. 4. Antagonism of BET proteins reduces MLV-integration frequencies at the transcription start sites. (*A* and *B*) Analysis of integration frequencies of ASLV, HIV-1, and MLV relative to BET proteins (Brd2, -3, and -4) or HP1α/β chromatin sites in HEK293T cells. The chromatin sites and promoters bound by BET proteins or HP1α/β were quantified using ChIP-Seq data (21). (*A*) Heatmap depicting association of integration sites with BET proteins or HP1α/β chromatin sites. (*B*) Heatmap depicting association of integration sites with promoters bound by BET proteins or HP1α/β. The frequency of integration sites relative to the matched random controls was quantified using the receiver operating characteristic area method (44). The color key depicts enrichment or depletion of chromatin sites or promoters bound by indicated protein near integration sites. All comparisons of MLV to HIV or ASLV achieved $P < 0.001$ (Wald statistic). (*C*–*F*) Percentage of MLV-integration sites found within each interval surrounding RefSeq transcription start sites (TSSs) in HEK293T cells. The integration sites near TSSs were compiled onto a single start site, and the frequencies were mapped. The *x* axis depicts the distance (in kb) relative to the TSSs (set at 0). The *y* axis depicts the percentage of integration sites in the indicated window. For comparison, integration sites of HIV-1 (9) and MLV (45, 46) in HEK293T cells are shown. (*C*) Dose-dependent effect of JQ-1 on MLV-integration frequencies at the TSSs. Percentage of MLV-integration sites within each interval surrounding TSSs in HEK293T cells treated with indicated concentrations of JQ-1 inhibitor or DMSO (indicated as "0 nM JQ-1"). (*D*) Percentage of MLV integration within 2-kb distance from TSSs. All samples achieved statistical significant (***$P < 0.001$; Fisher's exact test) compared with 0 nM JQ-1 treatment. (*E*) Effect of concurrent down-regulation of BET proteins on MLV-integration frequencies at the TSSs. Percentage of MLV-integration sites within each interval surrounding TSSs in HEK293T cells transfected with scrambled siRNA (indicated as "Sci") or a pool of Brd2, -3, and -4 siRNAs [indicated as "Brd(2+3+4)i"]. (*F*) Percentage of MLV integration within 2-kb distance from TSSs. Brd(2+3+4)i achieved statistical significant (**$P = 0.009$; Fisher's exact test) compared with Sci.

have adopted different chromatin-binding tethers to effectively integrate viral DNAs into specific features of chromatin.

BET proteins have been implicated in numerous aspects of medicine such as cancer, inflammation, obesity, and HIV latency (26–28, 30, 31, 39). Most relevant to this study, Brd4 acts as an attachment site for the tethering of papilloma viral genomes to the mitotic chromosomes. The E2 protein of bovine papilloma virus (BPV) tethers the viral genome to mitotic chromosomes via bimodal interaction of its C-terminal DNA-binding domain with viral DNA and the N-terminal transactivation domain with the

C-terminal region of Brd4 (40, 41). This E2–Brd4 interaction plays a critical role for BPV genome segregation by ensuring that the BPV episomal DNA is retained in the nucleus after cell division.

In this report, we have uncovered a role for BET proteins in promoting and targeting MLV integration to transcription start sites. These findings will facilitate the development of safer MLV-based vectors for human gene therapy. For example, the inclusion of the experimental drug JQ-1, in combination with MLV-based vectors, could minimize the risks of insertional activation of protooncogenes.

Materials and Methods

MS-Based Proteomic Analysis. MS experiments and data analysis were performed as described previously (42). See *SI Materials and Methods* for more details.

In Vitro Pull-Down Assays. Pull-down assays and determination of apparent K_d values were performed as described (29, 36).

Strand-Transfer Assays. The in vitro-concerted integration and homogeneous time-resolved fluorescence (HTRF)-based strand-transfer assays were carried out as reported previously (29, 43), with minor changes. See *SI Materials and Methods* for more details.

Isolation of Integration Sites. Isolation and sequencing of MLV-integration sites were performed as described previously (34), with some changes. See *SI Materials and Methods* for more details.

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