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***J. Virol.* 1999, 73(12):10406.**

JOURNAL OF VIROLOGY, Dec. 1999, p. 10406–10415 Vol. 73, No. 12
0022-538X/99/$04.00+0

Increased Induction of Osteopetrosis, but Unaltered Lymphomagenicity, by Murine Leukemia Virus SL3-3 after Mutation of a Nuclear Factor 1 Site in the Enhancer

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Received 5 May 1999/Accepted 8 September 1999

SL3-3 is a murine leukemia virus which is only weakly bone pathogenic but highly T-cell lymphomagenic. A major pathogenic determinant is the transcriptional enhancer comprising several transcription factor binding sites, among which are three identical sites for nuclear factor 1 (NF1). We have investigated the pathogenic properties of NF1 site enhancer mutants of SL3-3. Two different mutants carrying a 3-bp mutation either in all three NF1 sites or in the central site alone were constructed and assayed in inbred NMRI mice. The wild type and both mutants induced lymphomas in all mice, with a mean latency period of 9 weeks. However, there was a considerable difference in osteopetrosis induction. Wild-type SL3-3 induced osteopetrosis in 11% of the mice (2 of 19), and the triple NF1 site mutant induced osteopetrosis in none of the mice (0 of 19), whereas the single NF1 site mutant induced osteopetrosis in 56% (10 of 18) of the mice, as determined by X-ray analysis. A detailed histological examination of the femurs of the mice was carried out and found to support this diagnosis. Thus, the NF1 sites of SL3-3 are major determinants of osteopetrosis induction, without determining lymphomagenesis. This conclusion was further supported by evaluation of the bone pathogenicity of other SL3-3 enhancer variants, the lymphomagenicity of which had been examined previously. This evaluation furthermore strongly indicated that the core sites, a second group of transcription factor binding sites in the viral enhancer, are necessary for the osteopetrosis induction potential of SL3-3.

Murine leukemia viruses (MLVs) induce various diseases when injected into newborn mice of susceptible strains. Most common is the induction of hematopoietic neoplasia, but skeletal diseases such as osteopetrosis and osteomas are also encountered. Whereas osteomas are benign bone tumors (26), osteopetrosis is a generalized disorder of the skeleton (30). In contrast to avian leukosis virus-induced osteopetrosis in birds (53), MLV-induced osteopetrosis appears radiologically as a thickening of the cortex along the endosteal surface and a progressive increase in trabecular bone mass, maintaining the overall shape of the affected skeleton. In severe cases, the bone marrow cavity is completely filled with excessively accumulated bone (30).

SL3-3 is an ecotropic MLV of the Akv family. It is strongly T-cell lymphomagenic; however, a bone-pathogenic potential, as shown for Akv (26, 30, 48), has not been described as a characteristic feature of this virus. When inoculated into susceptible newborn mice, it induces malignant T-cell lymphomas within 2 to 4 months in most mouse strains (10, 12, 24, 54). Reports of other types of diseases induced by SL3-3 are scarce. In CBA mice, SL3-3 was recently reported to induce osteomas in 3 of 12 mice (38), whereas in NMRI mice infected with SL3-3, 2 of 27 mice were found to have osteopetrosis and none were found to have osteomas at the time of lymphoma development (42). This is in contrast to results for RFB MLV, another member of the Akv family closely related to SL3-3 and the most bone-pathogenic MLV described so far (16). RFB MLV induced osteomas in 100% of CBA/Ca strain mice (43). In NMRI mice, RFB MLV induced osteopetrosis in 60%, osteomas in 15%, and lymphomas in 90% of the infected mice (16).

Genomic regions critical for the oncogenic potential of many MLVs have been mapped to the transcriptional enhancer in the U3 region and even to individual binding sites therein. The enhancer in SL3-3 is comprised of 72 bp directly repeated one and a half times. Binding sites for at least six different classes of transcription factors have been characterized within this 72-bp region, and the role of most of these has been tested in pathogenicity studies of viruses with specific mutations introduced into the enhancer. The results thereof have shown sites of primary importance to be a Myb site (35) and the core site, which binds members of the AML1 transcription factor family (18, 29) (also known as CBF, PEBP2, and SEF1). A second site for this factor, the core site II, was found to be important only when the core site was simultaneously mutated (11, 18). Further, an Ets site, present in the enhancer, was found to be of minor importance (35) and a nuclear factor 1 (NF1) site was found to be dispensable for lymphomagenicity (10). An overlapping binding site for the glucocorticoid receptor (6) and basic helix-loop-helix transcription factors (33, 34) also exists,

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but its role in pathogenesis has not been examined. An alteration in the incidence of bone-related diseases has not been reported for enhancer mutants of SL3-3 or related MLVs, but other regions in the SL3-3 genome may play a role therein, since chimeras between SL3-3 and RFB MLVs pointed to a bone-pathogenic potential of non-long terminal repeat (LTR) regions (38).

The enhancer of SL3-3 contains an NF1 site, which, due to the repeat structure, is present three times. The site is part of a highly conserved region of the MLV U3 region and is found in an identical or nearly identical version in the majority of MLV enhancers (17). The NF1 family of transcription factors is characterized by distinct N-terminal DNA binding and dimerization domains and divergent C-terminal proline-rich transactivation domains (22, 28). NF1-encoding cDNAs have been cloned from several animals, identifying four different genes that are conserved between mammals and chickens (15, 21, 27, 40, 45, 46). NF1 binds its consensus sequence as a dimer, and since many splice variants are produced from the genes and heterodimerization occurs, a large number of NF1 complexes with different potentials may exist (7, 22, 25). NF1 has been shown to activate (4, 5, 13, 44) as well as repress (1, 20) the transcription of several cellular and viral genes and to act in a cell-specific manner (4, 14, 31). Details of molecular interactions between NF1 factors and the NF1 sites in the SL3-3 enhancer are not known, and the possibility remains that factors distinct from NF1 interact with the site also.

We have been interested in the NF1 sites in the SL3-3 enhancer because we previously noted that small regions in the enhancer repeats containing these sites were occasionally deleted in the process of tumor induction by viruses which had a reduced pathogenicity because they carried introduced mutations in the core (AML1) sites (10). These deletions of NF1 site regions together with other enhancer alterations were shown to result in a partial restoration of lymphomagenic potential and apparently were selected for as compensations for the introduced core site mutations (11, 12). Consistent with this, a mutation in all three NF1 sites in the SL3-3 enhancer did not impair the oncogenicity of the virus (10), although a mutation in the equivalent sites in the enhancer of Moloney MLV did result in a significantly prolonged disease latency period (50).

In this study, we have investigated the pathogenicity of an SL3-3 virus with a mutation in the central of the three NF1 sites and compared it to that of a virus with all three sites mutated. These mutants were found to have very similar lymphoma induction potentials but to differ markedly in their abilities to induce osteopetrosis.

MATERIALS AND METHODS

Cell lines. MB1.8 osteoblast-like cells, which support osteoclastogenesis (57) (generously provided by G. A. Rodan, Merck Inc., West Point, Pa.) were grown in αMEM (Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum, 2% L-glutamine, 100 U of penicillin, and 100 μg of streptomycin per ml. The murine osteoblastic MC3T3 (51) and KM1/K3 cell lines, described earlier (56), were grown in Dulbecco's modified Eagle's medium with *N*-acetyl-L-alanyl-L-glutamine (Seromed; Biochrom) supplemented with 10% fetal serum and antibiotics as above.

Generation of viruses. Generation of the wild-type SL3-3 virus and the SL3(3mNF1) virus variant has been previously described (10). The SL3(1mNF1) virus variant was generated in a similar manner. Briefly, the *Pst*I-*Kpn*I fragment of construct pSL3(3mNF1)cat (10) was inserted into a plasmid carrying the molecular clone of SL3-3, thereby replacing the wild-type sequence, and infectious viruses were produced by transfection into NIH 3T3 cells. Virus production was monitored by reverse transcriptase assays as described previously (18), and the LTR regions of proviruses were amplified from virus-producing cells by PCR and sequenced (see below) to confirm the integrity of the mutations.

Pathogenicity experiments. Inbred NMRI strain mice which lack ecotropic endogenous proviruses (23) were injected with 10^6 to 10^7 infectious virus particles within 36 h after birth. Control mice were mock injected with complete medium. The mice were checked for tumor development 5 days per week. The mice were killed when they showed signs of illness or tumor development. A complete necropsy, including X-ray analysis, was performed as described previously (47). Tumors were diagnosed on the basis of gross appearance of lymphoid organs and according to the cytological and anatomic criteria described by Pattengale (41). For histological diagnosis of osteopetrosis, sections of the distal femur were prepared and stained with hematoxylin and eosin and von Gieson stains.

PCR amplification of proviral DNA. DNA was prepared from frozen tumor material as described earlier (10). PCR was performed with a primer set recognizing the 5′ end of the U3 region and an SL3-3-specific sequence outside the LTR in the 5′ untranslated region (10). PCR products were purified using Dynabeads (Dynal M-280), and the U3 regions were sequenced using an automatic DNA sequenator (Applied Biosystems).

Transfections and reporter assays. Osteoblastic and fibroblastic cell lines were transfected by the calcium phosphate method as earlier described (10). A 3.0-μg amount of each chloramphenicol acetyltransferase (CAT) reporter plasmid was used. As reference plasmids for transfection efficiency, either 1.0 μg of pCH110 (*lacZ* gene driven by the simian virus 40 early promoter), p0IV249 (*lacZ* gene driven by the cytomegalovirus promoter), or pRSV-LUC (luciferase gene driven by the Rous sarcoma virus LTR) was used. The pUC19 or pBluescript plasmid was used as carrier DNA. Cat assays were done by the original thin-layer chromatography method with ^{14}C-labeled chloramphenicol using a PhosphorImager (Fujix Bas 1000) to quantitate the chromatograms, as earlier described (10). β-Galactosidase activity was measured using an *o*-nitrophenyl-β-D-galactopyranoside assay, and luciferase activity was measured with a luminometer (Berthold LB 9501) as described previously (10). All transfections were done in duplicate and repeated two to four times.

Plasmids. pSL3(3mNF1), pSL3(1mNF1), pSL3(2Δ18-3 1/2), and pSL3(2Δ18-2 1/2) were constructed on the basis of the constructs pSL3(3mNF1)-cat, pSL3(1mNF1)-cat, pSL3(Δ18+72)-cat, and pSL3(Δ18)-cat (10), respectively, by deleting a 288-bp *Sma*I-*Bgl*II fragment. Plasmids pSL3(wt), pSL3(dm), pSL3(TUMdm), and pSL3(atc) are identical to the previously described (11) plasmids pSL3(wt)-PBScat, pSL3(dm)-PBScat, pSL3(TUMdm)-PBScat, and pSL3(atc)-PBScat, respectively. Plasmid pRFB(wt)-PBScat was constructed on the basis of the RFB-14 molecular clone (43). All the CAT constructs contain the part of the LTR region which extends from the *Pst*I site in the 5′ end of the U3 region and into the *Sma*I site (SL3 constructs) or adjacent *Kpn*I site (RFB construct) of the R region.

RESULTS

Unaltered lymphoma induction by both single and triple NF1 site SL3-3 mutants. SL3-3 contains an NF1 site in the region of the enhancer that is directly repeated three times (Fig. 1A). Previously, we used transient transcription assays to test enhancer-promoter reporter constructs carrying an identical 3-bp mutation in each of the sites (10). This 3-bp mutation was known from band shift assays to disrupt binding of a nuclear complex believed to be NF1 (36). Compared to expression of the wild-type SL3-3 reporter, the reporter with three NF1 site mutations (3mNF1) had a twofold-increased level of expression in a T-cell line, whereas it had a fivefold lower expression in NIH cells, indicating a negative regulation by the site in the target cells of SL3-3 and a positive function in fibroblasts (10). To investigate if this effect relied on all three NF1 sites, a reporter construct was made in which only the central of the three sites carried the mutation (Fig. 1A). This mutant, 1mNF1, also gave a twofold-increased expression in the T-cell line but did not reduce expression in fibroblasts relative to that of the wild-type construct (10). This suggested that these two different enhancer mutants of SL3-3 might also have different pathogenic properties in nonhematopoietic tissues.

In order to test this possibility, we generated infectious SL3-3 virus particles containing the 1mNF1 mutation, i.e., the 3-bp mutation in the NF1 site in the central enhancer repeat (Fig. 1A). This mutant, denoted SL3(1mNF1), along with the previously described mutant SL3(3mNF1) (10), which contains the mutation in all three NF1 sites, was injected into newborn inbred NMRI mice, together with wild-type SL3-3 virus as a control. Both mutants and wild-type SL3-3 induced lymphomas in all injected mice, with mean latency periods of 61 to 64

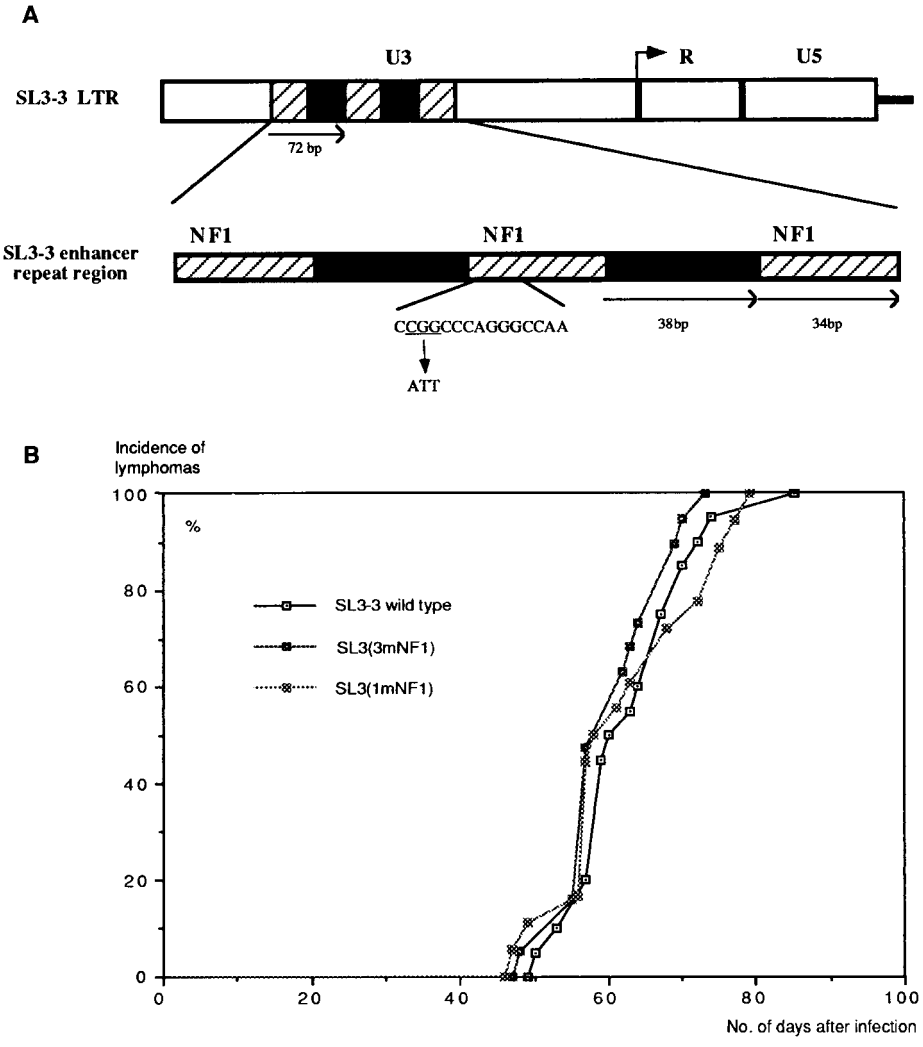


FIG. 1. (A) Schematic outline of the SL3-3 LTR region. The transcriptional enhancer is found within a part of the U3 region that consists of 72 bp which are repeated one-and-a-half times. The three identical NF1 sites are indicated, and the CGG to ATT mutation used in this study is also shown. SL3(3mNF1) carries the mutation in all three NF1 sites, whereas SL3(1mNF1) carries the mutation in the central site only. (B) Lymphoma incidence in inbred NMRI mice infected with the wild type or the two different NF1 site mutants of SL3-3. Mock-infected control mice did not develop lymphomas within the 100-day observation period. Prolonged observation of the control mice showed one mouse with a lymphoma at the age of 321 days and five more mice with lymphomas between 12 and 20 months.

days (Fig. 1B and Table 1). Histological examination of the tumors showed that they were all large cell lymphoblastic lymphomas, as found in previous experiments with SL3-3 (3, 18), and so the lymphomas induced by the mutants did not differ from the ones induced by wild-type SL3-3.

The U3 regions of proviruses from the tumor tissues were amplified by PCR and sequenced. The mutations of the NF1 sites were intact in all cases. Rearrangements of the enhancer regions were not found, apart from the variations in the number of enhancer repeats frequently observed in lymphomas induced by SL3-3 and enhancer variants thereof (10–12, 29, 35). The relative number of tumors harboring proviruses with an altered number of enhancer repeats did not appear to differ among tumors induced by the two mutants or the wild type. In the tumors induced by SL3(1mNF1), additional repeats in the proviral enhancer regions were found both with and without the mutation (data not shown).

These data indicate that the two mutants induce large cell lymphoblastic lymphomas with similar thymic involvement and with the same latency period as wild-type SL3-3. The data confirm and extend previous examinations of the lymphomagenic properties of the 3mNF1 mutant in random-bred NMRI mice (10). In addition, they show that a mutation of only the central of the three NF1 sites does not alter the lymphomagenic properties of the virus. It is interesting to note that the lymphoma latency period of wild-type SL3-3 and SL3(3mNF1) was considerably shorter in inbred NMRI mice, as shown here, than in random-bred NMRI mice, as shown earlier (10).

NF1 site mutations affect osteopetrosis induction. Previous experiments have shown that MLVs closely related to SL3-3 induce osteopetrosis and osteomas together with lymphomas to various degrees (16, 26, 30, 48). In the present experiment, osteopetrosis was diagnosed both by X-ray and histological analysis. X-ray analysis was done using the increase of bone structure and loss of the marrow cavity as characteristics of osteopetrosis (26, 30). By this analysis, the number of mice with osteopetrosis after infection with the two different NF1 site mutants was found to differ markedly (Fig. 2). Wild-type SL3-3 induced osteopetrosis in 2 of 19 mice (11%) (Table 1), confirming earlier data for osteopetrosis in SL3-3-infected

TABLE 1. Lymphoma and osteopetrosis in SL3-3 variant virus-infected NMRI mice

Virus variant	% Lymphoma incidence[a]	Latency in days (SD)	% of bone lesions identified by		
			X-ray	Histology[b]	
				Cancellous area	Cortical area
SL3-3	100 (19/19)	64 (8)	11 (2/19)	58 (11/19)	47 (9/19)
SL3(1mNF1)	100 (18/18)	63 (10)	56 (10/18)	94 (17/18)	83 (15/18)
SL3(3mNF1)	100 (19/19)	61 (7)	0 (0/19)	39 (7/18)	39 (7/18)
SL3(2Δ18-2 1/2)	100 (15/15)	68 (10)	7 (1/15)	75 (9/12)	67 (8/12)
SL3(2Δ18-3 1/2)	100 (16/16)	51 (5)	38 (6/16)	100 (10/10)	100 (10/10)
SL3(TUMdm)	100 (19/19)	121 (32)	0 (0/19)	11 (2/18)	0 (0/18)
SL3(dm)	80 (12/15)	248 (38)	0 (0/15)	36 (5/14)	29 (4/14)
SL3(atc)	100 (20/20)	144 (44)	0 (0/20)	39 (7/18)	6 (1/18)
Control[c]	4.8 (1/21)	ND[d]	0 (0/21)	0 (0/2)	0 (0/2)

[a] Tumor incidence within a 1-year observation period.
[b] Osteosclerotic lesion with active or inactive osteoblasts.
[c] Five mice developed spontaneous lymphomas between day 366 and day 580.
[d] ND, not determined.

mice (42). Surprisingly, 10 of the 18 mice (56%) infected with SL3(1mNF1), but none of the 19 mice infected with SL3(3mNF1), developed osteopetrosis (Fig. 2; Table 1). Osteomas were not detected in any of the infected or control mice.

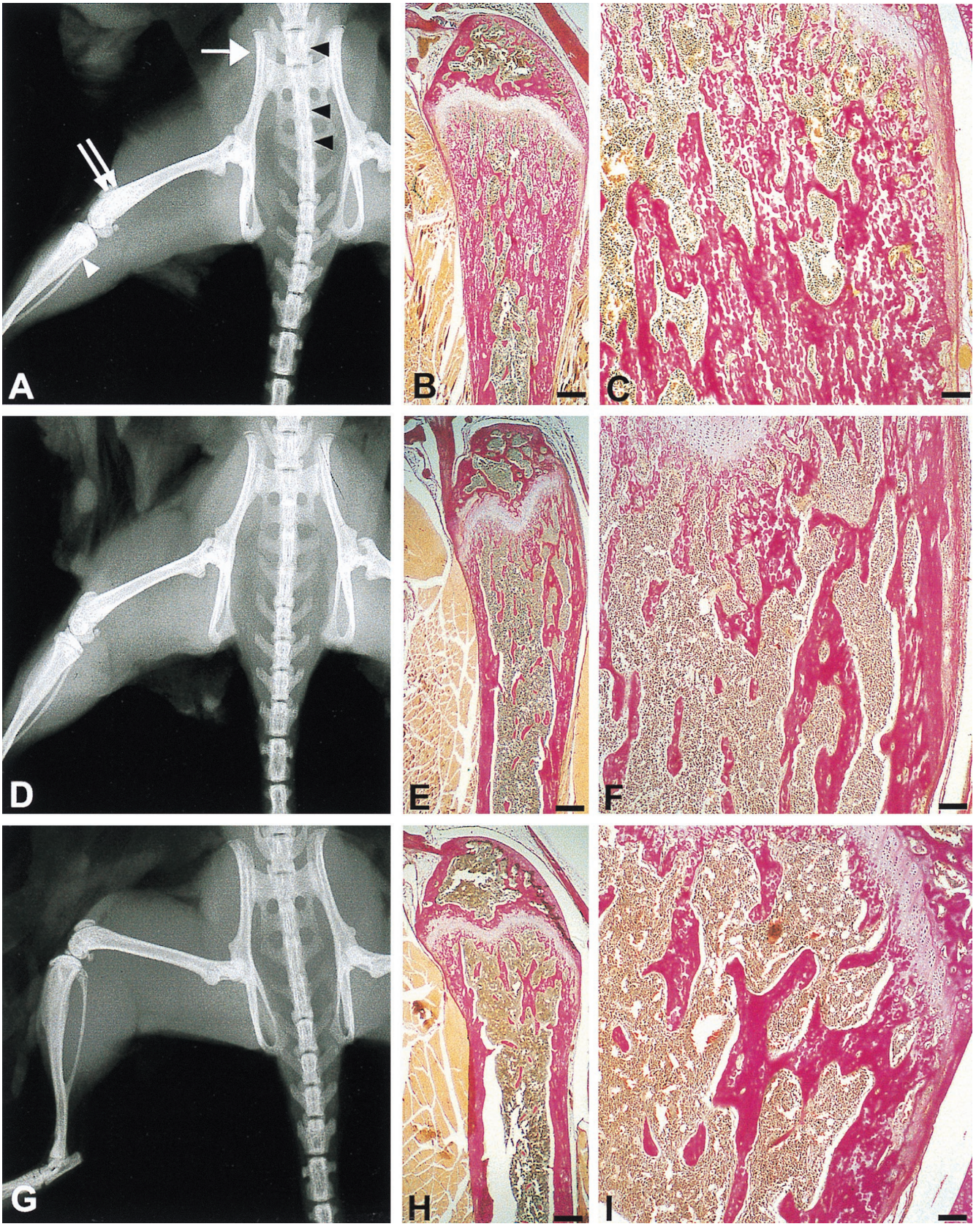
Recent histological and histomorphometric analyses of MLV-induced osteopetrosis have revealed that structural alterations can be detected in the skeleton at an earlier time point after infection, when these lesions are not as yet clearly detectable by X-ray analysis (49). Therefore, the distal femurs, one of the primarily affected sites of osteopetrosis, were analyzed histologically. Three main patterns were determined, including cancellous, cortical, and mixed lesions, with abnormal endochondral ossification as a common feature (Fig. 2). In the early phase of histopathological alterations, the lesions are primarily confined to the metaphyseal region, showing active bone deposition with a prominent osteoblastic layer surrounding the bone trabeculi. They progressively extended toward the diaphysis. Both cancellous and to a lesser extent cortical bone displayed histological alterations evolving into a more inactive stage, with absent osteoblastic activity and quiescent bone interfaces. Thus, the actual bone mass in advanced stages of osteopetrosis would depend on the dynamics of new bone formation during early stages. These findings point the bone lesions out as an evolving condition, which is clearly detectable by X-ray examination and with even higher sensitivity by histological analysis. Although the latter yielded higher levels of osteopetrosis incidence, the results of both methods were well correlated.

As was to be expected from the difference in sensitivity, bone lesions were detected histologically in mice injected with wild-type SL3-3 in the cancellous and in the cortical area with a diffuse pattern in 58 and 47% of the mice, respectively. In mice injected with the SL3(1mNF1) mutant, the incidence of cancellous and cortical lesions was 94 and 83%, respectively, whereas only 39% of the mice injected with SL3(3mNF1) showed alterations in these two regions (Table 1). In mock-infected control mice, one of 21 developed a lymphoma within a 1-year period and signs of osteopetrosis were not detected either by X-ray or histological analysis in any of the mice. These results show that an important determinant for osteopetrosis induction in the SL3-3 genome maps to the NF1 sites. Furthermore, they indicate that the potential of the virus to induce osteopetrosis is dependent on the exact number of intact NF1 sites or their relative positions in the enhancer.

Evaluation of the osteopetrosis induction potential of previously examined NF1 site enhancer variants. We have recently studied the lymphomagenicity of several SL3-3 enhancer variants with variations in the number of functional NF1 sites (10–12). It was therefore possible to analyze osteopetrosis incidence in mice infected with several other previously described SL3-3 enhancer variants.

First, the pathogenicity of SL3(3mNF1) and wild-type SL3-3 has been tested using random-bred NMRI mice (10). All mice developed lymphomas. None of 24 mice infected with SL3(3mNF1), but 1 of 49 mice infected with wild-type SL3-3, developed osteopetrosis, as determined by X-ray analysis (data not shown). This is consistent with the above-mentioned data indicating that wild-type SL3-3 has a limited potential to induce osteopetrosis, whereas such a potential has not been detected for the SL3(3mNF1) variant.

Second, two other SL3-3 enhancer variants, SL3(2Δ18-2 1/2) and SL3(2Δ18-3 1/2), that also differ by the number of intact NF1 sites have been shown to induce 100% lymphomas in inbred NMRI mice (12). Variant SL3(2Δ18-2 1/2) contains two identical deletions of 18 bp covering two of the three NF1 sites in the enhancer repeat region. This variant induced lymphomas with a latency period similar to that of wild-type SL3-3. Variant SL3(2Δ18-3 1/2) has an additional 72-bp repeat element and thus contains two intact NF1 sites in the enhancer region (Fig. 3). SL3(2Δ18-3 1/2) was found to induce lymphomas with a shorter latency period than wild-type SL3-3 (12). As diagnosed by X-ray analysis and shown in Table 1, SL3(2Δ18-2 1/2) induced osteopetrosis in one of 15 mice (7%), while SL3(2Δ18-3 1/2) induced osteopetrosis in 6 of 16 mice (38%). Whereas wild-type SL3-3 and SL3(2Δ18-2 1/2) induced osteopetrosis with similar incidences, the osteopetrosis potential of SL3(2Δ18-3 1/2) was considerably higher than those of wild-type SL3-3 ($P < 0.07$, Fisher's exact test) and SL3(2Δ18-2 1/2) ($P = 0.05$). The skeletal lesions were detectable histologically with similar differences in the incidence relative to X-ray analysis. Both cancellous and cortical areas of the femurs were affected in all the mice infected by SL3(2Δ18-3 1/2) and in 67 to 75% of the mice infected with the SL3(2Δ18-2 1/2) variant (Table 1). Whereas the bone lesions induced by SL3(2Δ18-2 1/2) were mainly quiescent, SL3(2Δ18-3 1/2)-induced lesions showed high osteoblastic activity (data not shown). These data again point to an involvement of the NF1 site region in determining the ability to induce osteopetrosis. The fact that SL3(2Δ18-3 1/2), like SL3(1mNF1), has only two functional



NF1 sites also supports the idea that disruption of some, but not all, of the NF1 sites of SL3-3 leads to an increase in the osteopetrosis induction potential.

Evidence that the core sites are required for osteopetrosis induction potential. Previous pathogenicity studies of the SL3-3 enhancer variants SL3(atc), SL3(dm), and SL3(TUMdm), showed that they induced lymphomas with high incidence after extended, variable latency periods in inbred NMRI mice (11). These variants carry different mutations in the core (AML1) sites, together with different numbers of functional NF1 sites (Fig. 3). None of the mice infected with these variants developed osteopetrosis (Table 1) as determined by X-ray analysis, even though lymphoma onset on average occurred after much longer periods of time than for the wild type. Histologically, they showed an incidence of cancellous and cortical lesions, comparable to those induced by SL3(3mNF1), except for variant SL3(TUMdm) which only induced cancellous lesions in 2 of 18 mice (Table 1).

It is particularly interesting to compare variants SL3(2Δ18-3 1/2) and SL3(TUMdm). Both contain two intact NF1 sites and both also contain an additional 72-bp repeat in combination with two deletions of either 18 bp [SL3(2Δ18-3 1/2)] or 28 bp [SL3(TUMdm)] of the two central NF1 site regions. However, SL3(TUMdm) also carries mutations of both core site I and core site II in all repeats (Fig. 3). Thus, these two variants are structurally quite similar with regard to the NF1 sites but differ by mutations of the core sites. SL3(TUMdm) has a very low potential to induce osteopetrosis (Table 1). In contrast, SL3(2Δ18-3 1/2) induced cancellous and cortical lesions in all infected mice to the extent that these were detectable by X-ray in 6 of 16 (38%) mice. The difference between these two variants is further emphasized by the fact that mice infected with SL3(TUMdm) had an average life span more than twice that of those infected with SL3(2Δ18-3 1/2). These findings strongly suggest that the core sites of the SL3-3 LTR represent a major determinant for osteopetrosis induction.

NF1 sites are important for enhancer strength in osteoblast cell lines. The mechanism of MLV-induced osteopetrosis is not as yet clear. Recent histomorphometric analyses of RFB MLV-infected NMRI mice showed significantly enhanced extracellular matrix production by infected osteoblasts. Furthermore, MLV infection of osteoblasts was also shown to interfere with recruitment and differentiation of osteoclast precursor cells (49), indicating a bifunctional effect of MLVs on cells of the osteoblastic lineage and subsequently on bone homeostasis. To examine the importance of the NF1 sites and to test whether induction of bone formation is associated with the strength of the viral enhancer in osteoblastic cell lines, we transfected SL3-3 enhancer CAT reporter constructs with the two types of NF1 site mutations into different cell lines of the osteoblastic lineage. Likewise, CAT constructs driven by the other above-mentioned SL3-3 enhancer variants and the RFB MLV enhancer were used and compared to the wild-type SL3-3 CAT construct. Three cell lines of different differentiation levels were used: MC3T3 osteoblast-like cells, MB1.8 stromal osteoblastic cells, and KM1/K3 osteoblast precursor cells.

The results of the experiments are shown in Table 2, in which the CAT activity of the wild-type SL3-3 constructs have been set to 100. The single NF1 site mutant construct pSL3(1mNF1) gave a two- to fourfold reduction in transcription levels relative to the wild-type construct, whereas the triple NF1 site mutant pSL3(3mNF1) gave a much stronger reduction in the transcription level of about 25-fold in each of the three cell lines. Thus, progressively mutating the NF1 sites progressively reduces the transcription levels, indicating that the NF1 sites play an important role in the expression of SL3-3 in osteoblasts. Turning to the constructs with deletions of NF1 sites instead of mutations, pSL3(2Δ18-2 1/2), which has one functional NF1 site, gave a two- to fourfold reduction and pSL3(2Δ18-3 1/2), which contains two functional NF1 sites plus an additional copy of the other transcription factor binding sites present in the enhancer repeats, gave transcription levels similar to those of the wild type. In the case of the three constructs that contain mutations in the core site regions, very different levels of reductions in transcription level were found for the three cell lines. In the osteoblast-like MC3T3 cells, the transcription rate was reduced 15- to 25-fold, whereas it was only around fivefold reduced in the stromal osteoblastic MB1.8 cells. In the KM1/K3 osteoblast precursor cell line, the level was only slightly reduced in the case of the two constructs with mutations in both core site I and core site II. This indicates that a transcription factor of importance for viral expression, which contacts the core sites, is present in the osteoblast-like MC3T3 cells and the stromal osteoblastic MB1.8 cells. In the osteoblast precursor KM1/K3 cells, this activity may be reduced or absent since the overall CAT activity level was rather low in this cell line (the wild-type level was low) compared to those of the MC3T3 and MB1.8 cells (data not shown). Finally, a construct in which the CAT gene is driven by the promoter-enhancer region from the potent bone-pathogenic RFB MLV (16) showed an up to twofold increase in transcriptional activity relative to the SL3-3 wild-type construct.

All in all, the transcription levels of the different enhancer variants in osteoblastic cell lines do not seem to correlate well with the osteopetrosis-inducing potential of the viruses. We note, however, that for all the viral variants that induce low levels of osteopetrosis (absent by X-ray analysis), the corresponding reporter constructs give rise to highly reduced (more than 10 times) levels of expression in MC3T3 cells, the osteoblast-like cell line.

FIG. 2. X-ray analyses and histological appearance of skeletal lesions of SL3-3 NF1 mutant virus-infected mice presenting with (A to F) and without (G to I) osteopetrosis. The roentgenographs show the areas in the skeleton which are primarily affected by osteopetrosis-inducing MLVs (30) and include the os ilium (arrow), lumbar vertebrae (black arrowheads), distal femur (double arrows), and proximal tibia (white arrowhead). (A) Characteristic roentgenographic appearance of an SL3(1mNF1)-infected, 61-day-old mouse (no. 364-705) with increased cancellous and cortical bone mass, indicative of osteopetrosis, as diagnosed by X-ray analysis. (B) Histological appearance of the growth cartilage/metaphyseal junction and cancellous area of the distal femur of the SL3(1mNF1)-infected mouse shown in panel A. Abnormal endochondral ossification and abundant deposition of new bone are shown in the cancellous and cortical areas in the trabeculi and along the cortex. (C) Higher magnification of panel B, showing thickening of the trabeculi and replacement of the marrow cavity by newly formed bone. (D) X-ray analysis as in the legend to panel A, but of an SL3(3mNF1)-infected, 48-day-old mouse (no. 369-706). The increased, roentgen-dense bone structures, compared to those in panel A, are below the stage of unequivocal diagnosis of osteopetrosis by X-ray analysis. (E) Histological section as described in the legend to panel B, but of the femur from the mouse displayed in panel D, showing increased bone mass at the cortical region of the distal femur. (F) Higher magnification of panel E, showing a similar disorganized growth zone as in the section shown in panel C but with a smaller number of bone trabecules extending into the marrow cavity and containing nonresorbed, mineralized cartilage. Note the virtual absence of trabecular coalescence but similar abnormal endochondral ossification of this femur as that shown in panel C. (G) X-ray analysis as described in the legend to panel A but of an SL3(3mNF1)-infected, 69-day-old mouse (no. 369-843) without signs of osteopetrotic lesions. Note the clearly defined cancellous and cortical bone structures in the lumbar vertebrae, os ilium, femur, and tibia. (H) Histological section as shown in panel B but from the femur of the mouse displayed in panel G. (I) Higher magnification of panel H, showing mature bone trabeculi and a nearly occluded growth zone, indicating a normal pattern of endochondral ossification. Van Gieson-stained sections; bars in panels B, E, and H, 854 μm; bars in panels C, F, and I, 204 μm.

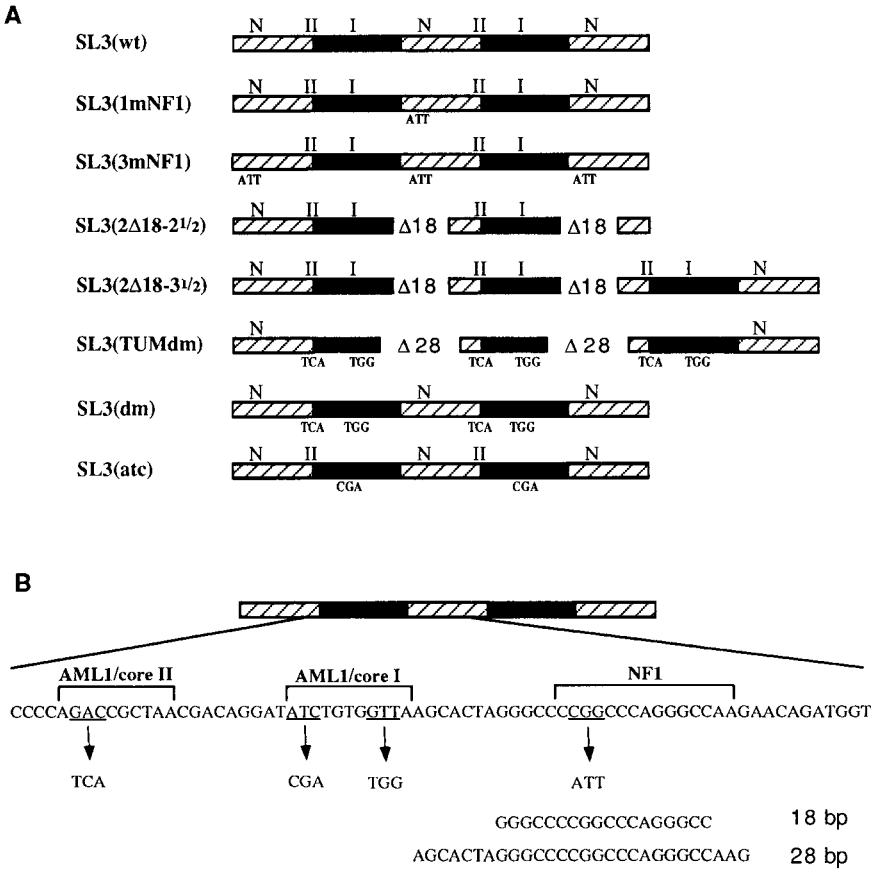


FIG. 3. Structure of the various enhancer variants of SL3-3. (A) Organization into 34- and 38-bp repeat elements is shown as hatched or filled boxes. The presence of 3-bp mutations is indicated below each structure, and deletions of either 18 or 28 bp are shown as gaps. The presence of intact binding sites is indicated above each of the structures: N, NF1 site; I, core site I; II, core site II. (B) The sequence of one 72-bp repeat element. The exact location of the various 3-bp mutations introduced into the enhancer and the 18-bp and 28-bp sequence deleted in some of the enhancer variants are shown.

DISCUSSION

In this study, we have built on the observation that two different NF1 mutants of the SL3-3 enhancer gave rise to different levels of transcriptional activity in different cell lines (10) and asked if this would be reflected in the pathogenicity of viruses having these mutations. We found that this was indeed the case: the two SL3-3 enhancer mutants show a dramatic difference in the potential to induce osteopetrosis. The two mutants differ by having mutations in either one or all three of the NF1 sites located in the enhancer repeat region. One of these mutants has a reduced ability to induce osteopetrosis relative to the wild type, whereas the other has an increased ability. The result not only indicates that the viral enhancer is an important genetic determinant in the process of MLV-induced osteopetrosis but directly points to the NF1 sites as playing key roles in this process. Furthermore, the two different enhancer mutants are interesting in that they have identical

TABLE 2. Transient transfection experiments in osteoblastic cell lines

Virus construct	Cell line (SEM)[a]			No. of NF1 sites	Presence of intact AMLI	Osteopetrosis potential of virus[b]
	MC3T3	MB1.8	KM1/K3			
pSL3(wt)	100	100	100	3	Yes	Intermediate
pSL3(1mNF1)	23 (3)	38 (5)	50 (5)	2	Yes	High
pSL3(3mNF1)	4.9 (0.3)	4.6 (2)	6.3 (2)	0	Yes	Low
pSL3(2Δ18-2 1/2)	29 (6)	35 (2)	48 (12)	1	Yes	Intermediate
pSL3(2Δ18-3 1/2)	92 (16)	105 (18)	83 (14)	2	Yes	High
pSL3(TUMdm)	4.2 (0.7)	19 (1)	81 (30)	2	No	Low
pSL3(dm)	3.0 (0.1)	18 (7)	82 (11)	3	No	Low
pSL3(atc)	7.1 (0.01)	13 (0.5)	20 (15)	3	No	Low
pRFB(wt)	184 (51)	231 (23)	182 (41)	2[c]	Yes	High

[a] The CAT activity level of the wild-type SL3-3 construct was arbitrarily set to 100 for each cell line.
[b] Summary of data from Table 1, with 0% osteopetrosis incidence (X-ray analysis) noted as low, ≤30% incidence as intermediate, and >30% incidence as high. In the case of RFB, the data are taken from reference 16.
[c] RFB MLV contains two additional NF1 sites of a different type (see reference 16).

lymphoma induction latency periods. MLV-induced osteopetrosis is a disease that up until now has always been associated with the development of lymphomas. When comparing the osteopetrosis induction potentials of different MLVs, one complicating factor has been the variations in lymphomagenicity of these viruses, since such differences may blur the mapping of osteopetrogenic genetic determinants. This problem is not encountered by the two mutants we describe in the present study. Assuming that the two NF1 site mutants induce lymphomas by an identical mechanism, they also demonstrate that MLV-induced osteopetrosis is not simply an epiphenomenon upon the induction of lymphomas. Since the osteopetrosis induction potential of the two mutants varies while the lymphomagenic potential is unaltered, the mechanisms of the two diseases must rely at least partly on different viral genetic elements.

We describe several SL3-3 enhancer mutants with widely varying osteopetrosis induction potentials. Taken together, the results indicate that the exact number of NF1 sites present in the enhancer is critical for the ability of SL3-3 to induce osteopetrosis. The two variants with increased osteopetrosis induction potential, SL3(1mNF1) and SL3(2Δ18-3 1/2), both contain two intact NF1 sites in the enhancer repeats. In contrast, versions of SL3-3 containing three (wild type) or one [SL3(2Δ18-2 1/2)] functional NF1 site in the repeat region induced a low incidence of osteopetrosis, whereas SL3(3mNF1), which lacks functional NF1 sites, did not induce roentgenographically detectable lesions. It is interesting to note that RFB, the MLV which has the strongest reported osteopetrosis induction potential (30), also contains two NF1 sites in its enhancer repeat region (16).

The mechanism by which the NF1 sites participate in the mechanism of osteopetrosis development remains unclear. One possibility is that the 3-bp mutation we have used creates a new transcription factor binding site or enhances the use of the 5′ half site which is not affected by the mutation. However, the fact that variant SL3(2Δ18-3 1/2), which does not contain the 5′ half site since it carries deletions of the NF1 sites instead of mutations, also has an increased ability to induce osteopetrosis argues against this possibility. Another more likely mechanistic explanation is that the mutation of the central NF1 site disrupts the binding of one or more factors to the site. This would mean that binding of a factor or complex to the central NF1 site has a down-regulatory effect on enhancer function.

Both activating and repressing functions of NF1 are known, and even instances in which the same enhancer is variously repressed or activated by NF1 complexes depending on cell type have been described (4, 14, 25). It is therefore not unlikely that NF1 complexes act to repress the SL3-3 enhancer in some cell types and to activate it in others. This could help to explain why both zero and three NF1 sites in the viral enhancer result in low osteopetrosis induction, while two sites give a high induction. If the virus in order to induce osteopetrosis has to be expressed in several cell types for which NF1 activates transcription in some and represses it in others, it is likely that there is an optimal number of binding sites that would result in the most efficient overall expression. In the present case, it would mean that in order for osteopetrosis induction to take place, two sites are needed for the virus to be sufficiently expressed in cell types for which NF1 is an activator, whereas three sites inhibit the expression too much in cell types for which NF1 is a repressor. This hypothesis would also predict that the cell type(s) in which NF1 acts as a transcriptional repressor has to be infected to facilitate induction of osteopetrosis but not of lymphomas.

Another possible explanation for the negative effect of the central NF1 site is that the factor(s) binding to it does not actively repress transcription but instead simply interferes with the action of other factors that contact nearby binding sites. This idea comes to mind because the binding sites in the enhancer of SL3-3 are positioned very tightly. It is therefore tempting to assume that all sites cannot be occupied simultaneously and that a competition for binding sites exists. Furthermore, some of the interacting factors, such as AML1 (CBF), Myb, and Ets factors, are known to contact each other on the enhancer and to transactivate transcription in a cooperative manner (19, 52, 58–60) and occupancy of the central NF1 site may therefore disrupt interactions between such factors.

The NF1 sites seem important for the viral enhancer in cell types implicated in osteopetrosis development. The function of MLVs in MLV-induced osteopetrosis is believed to be stimulation of the bone-modeling actions of osteoblasts as well as the impairment of bone resorption through reduced recruitment and differentiation of osteoclasts (49). In this study, we have focused on the action of the SL3-3 enhancer in osteoblasts. Transient transfection experiments of reporter constructs into three osteoblastic cell lines at different differentiation levels showed a clear correlation between the number of NF1 sites and the enhancer strength. This result underlines the importance of the NF1 sites for the expression of SL3-3 in these cell types. Assuming a more or less linear relationship between enhancer strength and viral pathogenicity does not, however, explain why the enhancer variants with only two intact NF1 sites have a higher osteopetrosis induction potential than wild-type SL3-3. There may be several reasons for this. One possibility is that the osteoblast cell lines in which we tested the enhancers are not representative of all the cell types that need to be targeted by SL3-3 in vivo. In line with the discussion above, it is conceivable that SL3-3 needs to infect a cell type in which NF1 acts as a repressor. This cell type is not represented by the panel of osteoblastic cell lines we have used, but it may still very well be a bone cell. Interestingly, osteoclasts were recently found to be among the bone marrow cells directly infected by Moloney MLV after intraperitoneal injection in a study using a replication-defective Moloney MLV-based vector (37). Another possibility is that transient transfection experiments do not allow for the examination of all aspects of NF1 behavior. In particular, the chromatin structure of the integrated provirus is unlikely to be mirrored in transfection experiments. This may be important since NF1 has been linked to the dynamic conduct of the nucleosome structure. For instance, NF1 interactions on the mouse mammary tumor virus LTR have been shown to be mediated in part by the nucleosome structure (5, 8) and NF1 has been found to directly interact with histone H3 in core particles (2).

The pathogenicity results indicate that NF1 sites are implicated in osteopetrosis development but not in the process of lymphoma induction. Evaluation of the osteopetrosis induction potential of the core mutants indicates that the reverse is not true for the core sites. Mutation of the core sites greatly delays and also impairs lymphomagenicity and at the same time also seems to abrogate osteopetrosis development. This conclusion is based on two observations. First, none of the three SL3-3 variants with core site mutations induced osteopetrosis at the X-ray level, and histologically, skeletal lesions were only found with a low incidence, even though the mice lived considerably longer than mice infected with the wild type. Second, there was increased osteopetrosis induction by SL3(2Δ18-3 1/2) and a lack of osteopetrosis induction by SL3(TUMdm), two variants that are structurally similar except for core site mutations. The latter variant contains mutations in both core site I and site II in all three repeat elements, whereas the former has intact core

sites. However, SL3(2Δ18-3 1/2) contains NF1 site deletions of 18 bp, whereas the deletions present in SL3(TUMdm) are of 28 bp. The conclusion therefore relies on the premise that the 18-bp and 28-bp deletions function in an identical manner. This seems to be a fair assumption since the larger deletion removes a complete turn of the DNA helix and does not appear to affect any of the known neighboring binding sites in the enhancer. Furthermore, both types of deletions originally arose in tumors induced by core mutants of SL3-3 and are thought to compensate for their reduced tumorigenicity by the same mechanism (10).

Why do the core site mutations affect the ability of SL3-3 to induce osteopetrosis? MLV-induced osteopetrosis is a condition which may be coupled to the development of lymphomas. Therefore, it is possible that the reduced lymphomagenicity of the core site mutants could account for the lack of osteopetrosis development; i.e., the core site mutations may interfere with the steps in lymphoma development that are necessary for the development of osteopetrosis. However, it seems more likely that the core site mutations interfere directly with the development of osteopetrosis independently of lymphoma development, since the core site mutants after all were quite lymphomagenic. Thus SL3(TUMdm), the variant with the lowest osteopetrosis induction potential, induced lymphomas in all mice with a mean latency of only 4 months. This indicates that the core sites are contacted by a transcription factor that is important in bone cells. The transient transfection experiments performed here, using the three different SL3-3 reporter constructs with mutant core sites, do show a highly reduced activity in two of the osteoblastic cell lines used, indicating that this may indeed be the case. A possible candidate for such a factor would be CBFA1 (also denoted as AML3 and PEBP2aA), a member of the core binding factor family. This factor was recently found to be required for the development of osteoblasts and is believed to be a major developmental switch of this cell type (9, 39). It may be expected to be able to activate the transcription of SL3-3 through the core sites in osteoblasts in the same way as its family member AML1 does in T cells (55, 61). In agreement with this idea, CBFA1 has been found to be expressed in MC3T3 and MB1.8 cells, but is expressed at a very low level in KM1/K3 cells (32), the osteoblastic precursor cell line in which we did not detect reduced transcription levels with the core site mutants.

In summary, we have established the viral enhancer as a bone pathogenic determinant and have shown that the NF1 sites of the SL3-3 enhancer are key determinants of osteopetrosis induction without affecting the lymphoma induction potential. Also, we have presented strong evidence that the core sites are necessary for osteopetrosis induction. Furthermore, we find that the number of NF1 sites in the SL3-3 enhancer is critical for osteopetrosis induction, with two being the optimal number, arguing that the NF1 sites are implicated in both negative and positive regulation.

ACKNOWLEDGMENTS

We thank Angelika Appold, Lone Højgaard, Jacqueline Müller, Anna Nickl, and Elenore Samson for excellent technical assistance.

This work was supported by the Danish Cancer Society, the Karen Elise Jensen Foundation, the Danish Natural Sciences Research Council, The Danish Biotechnology Program, and the Leo Nielsen Foundation and by European Commission contracts CT-950100 (Biotechnology), CT-950675 (Biomed-2), and CT95-0008 (Nuclear Fission Safety). S.E. was supported in part by a DAAD fellowship.

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