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Differential and Common Leukemogenic Potentials of Multiple *NUP98-Hox* Fusion Proteins Alone or with Meis1

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NUP98-Hox **fusion genes are newly identified oncogenes isolated in myeloid leukemias. Intriguingly, only *Abd-B Hox* genes have been reported as fusion partners, indicating that they may have unique overlapping leukemogenic properties. To address this hypothesis, we engineered novel NUP98 fusions with Hox genes not previously identified as fusion partners: the *Abd-B*-like gene *HOXA10* and two *Antennepedia*-like genes, *HOXB3* and *HOXB4*. Notably, *NUP98-HOXA10* and *NUP98-HOXB3* but not *NUP98-HOXB4* induced leukemia in a murine transplant model, which is consistent with the reported leukemogenic potential ability of *HOXA10* and *HOXB3* but not *HOXB4*. Thus, the ability of *Hox* genes to induce leukemia as NUP98 fusion partners, although apparently redundant for *Abd-B*-like activity, is not restricted to this group, but rather is determined by the intrinsic leukemogenic potential of the Hox partner. We also show that the potent leukemogenic activity of *Abd-B*-like *Hox* genes is correlated with their strong ability to block hematopoietic differentiation. Conversely, coexpression of the Hox cofactor *Meis1* alleviated the requirement of a strong intrinsic Hox-transforming potential to induce leukemia. Our results support a model in which many if not all *Hox* genes can be leukemogenic and point to striking functional overlap not previously appreciated, presumably reflecting common regulated pathways.**

The clustered homeobox *Hox* genes encode a highly conserved family of transcription factors characterized by a 60-amino-acid DNA binding motif, the homeodomain. While *Hox* genes were first recognized for their prominent roles in embryonic development, their involvement in both normal and malignant hematopoietic processes is now well documented (2, 6, 30).

A central role for *Hox* genes in hematological malignancies is supported by the frequently observed elevation of *Hox* gene expression in acute myeloid leukemia (AML) (11, 14, 20) and the common involvement of their upstream regulator, *MLL*, in both myeloid and lymphoid leukemia (8). A more direct involvement for *Hox* genes in leukemia is supported by their frequent fusion to the nucleoporin gene *NUP98*. *Hox* genes are the most frequent fusion partner in an ever-growing number of *NUP98* fusion genes isolated almost exclusively in patients with myeloid leukemia (AML, posttherapy AML, and chronic myeloid leukemia). Reported Hox partners of *NUP98* include *HOXA9* (3, 21), *HOXD13* (27), *HOXA13* (9), *HOXA11* (9), *HOXC11* (35), and *HOXC13* (19). All NUP98-Hox fusions reported to date consist of the N terminus of NUP98, containing a region of multiple phenylalanine-glycine (FG) repeats that may act as a transcriptional coactivator through binding to CBP/p300 (13), and the C terminus of the *Hox* gene product, containing the intact homeodomain and various extents of flanking amino acids. The leukemogenic potential of such chimeric proteins was confirmed for *NUP98-HOXA9* (*NA9*) and *NUP98-HOXD13* (*ND13*) in retrovirally transduced murine bone marrow transplantation models with both genes inducing AML and/or a myeloproliferative syndrome (16, 25). The transforming potentials of NA9 and ND13 were found to be dependent on the ability of their homeodomains to bind DNA (13, 25), and the Pbx interacting motif of HOXA9 in NA9 was found to be dispensable, suggesting that the Hox DNA-binding domain is the minimal and possibly only significant contribution of the Hox fusion partner.

The leukemogenic activity of *Hox* genes is not restricted to chimeric NUP98-Hox proteins, since *HOXB8, HOXA10*, *HOXB3*, and *HOXA9* have previously been shown to induce AML upon engineered overexpression (15, 24, 31, 37). This suggests that a key result of NUP98 fusion with Hox is deregulated expression of Hox-responsive genes by the chimeric Hox protein, in which NUP98 replaces the transcriptional activity of the Hox N-terminal region (13). Consistent with this, both *HOXA9* and *NA9* studied in similar experimental settings are leukemogenic and block myeloid progenitor differentiation in interleukin-3 and granulocyte-macrophage colony-stimulating factor (7, 16). Nevertheless, differences in disease latency, cooperativity with Meis1, and other in vitro growth properties suggest that NUP98-Hox fusions have different properties compared to their normal counterparts. For instance, in vitro, *HOXA9* did not replicate the effect induced by *NA9*, such as proliferation in stem cell factor and granulocytic differentiation block with granulocyte colony-stimulating factor (7).

Intriguingly, so far only *Hox* genes belonging to the Abd-B-like Hox paralog groups (groups 9 to 13) have been characterized as *NUP98* fusion partners, and within those, only members from paralog groups 9, 11, and 13 have been observed. Thus, it may be that only a subset of *Hox* genes, sharing potential overlap in gene targets and/or pathways, can complement *NUP98*. Outside of the *Abd-B*-like *Hox* genes, it is of interest

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that *HOXB4*, while having potent ability to enhance hematopoietic stem cell (HSC) expansion is on its own nonleukemogenic, whereas the near neighbor *HOXB3* can cause marked myeloproliferation and ultimately leukemia (1, 31). These findings raise the interesting question as to the intrinsic leukemogenic potential of *Hox* genes across the clusters and to their leukemogenic potential in the context of fusion genes.

The leukemic potential of intact *Hox* genes, such as *HOXB3* and *HOXA9*, and *NUP98* fusions with *HOXA9* or *HOXD13* has been shown to be increased by co-overexpression of *Meis1* (15, 22, 25, 36) as evidenced by decreased disease latency. The basis for this cooperativity is unclear because, although in vitro studies have suggested that *Meis1* expression can synergize with *Hox* genes in promoting a stronger differentiation block of Hox-transformed myeloid progenitors (5), it does not show leukemogenic activity on its own in vivo (16, 25). Understanding the role of *Meis1* is further complicated by the fact that several of the known collaborating Hox proteins involved lack the ability to physically interact with Meis1, as in the case of HOXB3 and ND13. Finally, it is not clear whether all NUP98-Hox fusions could be strongly complemented by *Meis1* since the leukemogenic potential of *NA9* was only weakly augmented by *Meis1*, whereas *Meis1* strongly complemented *ND13* (16, 25).

The principal objectives of the work described here were to test whether the preferential identification of *Abd-B*-like *Hox* genes as *NUP98* fusion partners in human leukemias is a consequence of their special intrinsic properties or, on the contrary, whether other *Hox* genes might share the functional capacity to form oncogenic *NUP98* fusion genes. The leukemogenic potential of various *NUP98-Hox* fusion genes representing *Hox* genes of different paralog groups and different leukemogenic potentials was tested in vivo, alone and in concert with *Meis1*, to enlarge the knowledge of the role of *Meis1* in Hox-related leukemia. Furthermore, in order to gain further insight into the mechanism(s) of Hox-mediated leukemia, we analyzed the impact of the native and *NUP98*-fused *Hox* genes on bone marrow differentiation and proliferation.

MATERIALS AND METHODS

Retroviral vectors and engineering of novel NUP98-Hox fusion genes. The murine stem cell virus (MSCV) Flag-NUP98–HOXD13 (ND13) internal ribosomal entry site (IRES)-enhanced green fluorescent protein (eGFP), MSCV ND13 IRES-eGFP, MSCV Meis1 IRES-yellow fluorescent protein (YFP), MSCV HOXB4 IRES-eGFP, and MSCV HOXA10 IRES-eGFP viral vectors used were previously described (1, 4, 25). We engineered the fusion of the cDNA portion corresponding to the homeobox-containing exon of *HOXA10* (NUP98-HOXA10; NA10), *HOXB4* (NB4), and *HOXB3* (NB3) with the *NUP98* portion found in the *ND13* fusion gene, which consists of exons 1 to 12 of *NUP98* (Fig. 1A). All gene fragments were obtained by PCR from cloned cDNAs with the Platinum *Taq* DNA polymerase High Fidelity (Invitrogen, Burlington, Canada); the primer sequences are available in the appendix. Constructs were validated by sequencing and correct expression and transmission were confirmed by Western blot and Southern blot analysis (Fig. 1B and C).

Mice and retroviral infection of primary bone marrow cells. Parental strain mice were bred and maintained at the British Columbia Cancer Research Centre animal facility. Donors of primary bone marrow cells were (C57BL/6Ly-Pep3b × C3H/HeJ)F_1 mice and recipients were (C57BL/6J × C3H/HeJ)F_1 (B6C3) mice. Primary mouse bone marrow cells were transduced as previously described (25). Briefly, bone marrow cells were harvested from mice treated 4 days previously with 150 mg of 5-fluorouracil/kg (Faulding, Underdaler, Australia) and stimulated for 48 h in Dulbecco's modified Eagle's medium supplemented with 15% fetal bovine serum, 10 ng of human interleukin-6 per ml, 6 ng of murine interleukin-3 per ml, and 100 ng of murine stem cell factor (StemCell Technologies

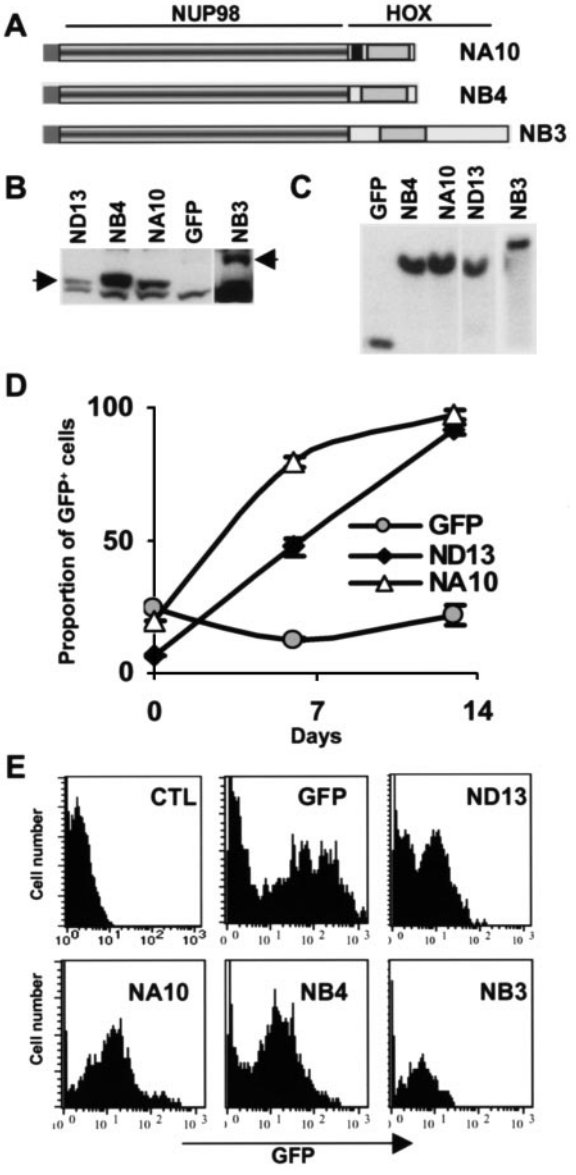

FIG. 1. Engineering of novel *NUP98-Hox* fusion genes. (A) Three new *NUP98-Hox* fusion genes were engineered by fusing the cDNA sequence corresponding to the homeobox-containing exon of *HOXA10* (NA10), *HOXB4* (NB4), and *HOXB3* (NB3) to that of *NUP98*. Only NA10 retained its Pbx-interacting motif, which is indicated as a black rectangle. The left grey boxes indicate the Flag tag, and the homeodomains are illustrated as light grey rectangles. (B) Western blot analysis of cell extracts from transduced BaF3 cells with a monoclonal Flag antibody. (C) Southern blot analysis showing full-length proviral integration of the various NUP98-Hox IRES-GFP viral vectors in transduced bone marrow cells. (D) *NA10* expression provides bone marrow cells with a growth advantage in vitro. The proportion of transduced GFP^+ bone marrow cells over time in liquid culture established with nonpurified transduced bone marrow cells is shown. Results of a representative experiment are shown: mean ± standard deviation of triplicate culture ($n = 3$). (E) Expression level of the GFP reporter gene in circulating leukocytes from mice transplanted with *NB4*, *NA10*, *NB3*, and *ND13* transduced bone marrow cells 8 to 13 weeks post-transplant. Histogram profiles of representative and GFP control (CTL) mice are shown.

TABLE 1. Engraftment levels in mice transplanted with singly transduced bone marrow cells 8 to 13 week posttransplant[a]

Gene transduced	% GFP$^+$ white blood cells ± SD	No. of blood cells	
		White (10^6/ml) ± SD	Red (10^9/ml) ± SD
GFP (control)	63.5 ± 11.5 (8)[b]	7.9 ± 1.5 (6)	10.8 ± 1.0 (7)
NA10	70.7 ± 5.9 (7)	7.7 ± 2.0 (7)	11.3 ± 1.6 (3)
NB4	69.5 ± 5.6 (10)	7.8 ± 1.3 (10)	9.8 ± 1.0 (9)
A10	50.7 ± 14.5 (7)	9.0 ± 3.6 (7)	10.7 ± 1.1 (3)
B4	63.6 ± 10.3 (6)	6.9 ± 1.3 (6)	9.1 ± 1.7 (6)

[a] Results shown are means from at least two independent cohorts.
[b] Total number of recipients analyzed.

Inc., Vancouver, Canada) per ml. All bone marrow cultures used medium. The cells were infected by cocultivation with irradiated (4,000 cGy of X-ray) GP+ E-86 viral producer cells with the addition of 5 μg of protamine sulfate (Sigma, Oakville, Canada) per ml.

A 1:1 mixture of producers of the NUP98-Hox viruses (with the green fluorescent protein [GFP] marker) and Meis1 virus (with the YFP marker) was used to coinfect bone marrow cells. Loosely adherent and nonadherent cells were harvested from the cocultures after 2 days and cultured for 48 h in the same medium without protamine sulfate. Where indicated, transduced cells were highly purified based on expression of GFP, YFP, or both fluorescent proteins with a FACS-Vantage (Becton Dickinson, Mississauga, Canada), as previously described (25).

Bone marrow transplantation and monitoring of recipients. Purified GFP$^+$ and/or nonpurified (unsorted cells) transduced bone marrow cells were injected into the tail vein of irradiated (900 cGy of ^{137}Cs γ-radiation) recipient F_1 B6C3 mice. Mice transplanted with singly transduced bone marrow cells received from 1.4×10^5 to 3.5×10^5 GFP$^+$ cells/transplant and mice injected with doubly transduced cells received from 0.2×10^4 to 8.0×10^4 GFP$^+$/YFP$^+$ bone marrow cells. For peripheral blood, bone marrow, and spleen cell analysis, single-cell suspensions were stained with the following monoclonal antibodies: phycoerythrin-labeled Gr-1, Mac-1, B220, Ter-119, and c-Kit (all obtained from Pharmingen, San Diego, Calif.). The immunoglobin E (IgE) receptor was detected with a polyclonal biotinylated anti-IgE receptor antibody (StemCell Technologies Inc.) followed by streptavidin-phycoerythrin (Pharmingen, San Diego, Calif.). Morphological analysis of peripheral blood, bone marrow, and spleen cells and histological analysis were performed as previously described (25).

In vitro assays. Cell proliferation was assessed in the same media described above. Differentiation of clonogenic progenitors was analyzed by plating cells in 1 ml of methylcellulose culture medium per petri dish in standard conditions (Methocult M3434, StemCell Technologies Inc.), containing 10 ng of murine interleukin-3 per ml, 10 ng of human interleukin-6 per ml, 50 ng of murine stem cell factor per ml, and 3 U of human erythropoietin per ml. Colonies were scored microscopically with standard criteria after 8 to 10 days.

CFU-S assay. GFP$^+$ purified bone marrow cells were first cultured for 7 days before injection. The total cell progeny from the starting equivalent (day 0) of 6 cells up to 1×10^6 cells were then injected into irradiated recipient mice. The recovery of CFU-spleen (CFU-S) cells was measured by determining the number of macroscopic colonies on the spleen at day 12 postinjection after fixation in Telleyesnickzky's solution.

Southern blot analysis. Genomic DNA was isolated with DNAzol reagent as recommended by the manufacturer (Invitrogen), and Southern blot analysis was performed as previously described (29). The stable integration of the provirus was confirmed by digesting the DNA with *Nhe*I (cutting in each long terminal repeat) and then the DNA-containing membrane was probed with ^{32}P-labeled GFP cDNA. For clonality analysis DNA from NUP98-Hox plus Meis1 primary or secondary transplant recipients was digested with *Eco*RI and probed with a ^{32}P-labeled NUP98 cDNA. DNA from *HOXB4*-plus-*Meis1*- and *HOXA10*-plus-*Meis1*-transplanted mice was digested with *Xho*I and *Eco*RI and hybridized to *HOXB4*- or *HOXA10*-specific probes, respectively.

Western blot analysis. Transduced BaF3 cells (infected with the same procedure as that for bone marrow) were lysed (400 mM NaCl, 0.1% Triton, 0.1 M HEPES, pH 7.5). Cell lysate was loaded onto a 10% Bis-Tris polyacrylamide gel (NuPAGE [NP0303], Invitrogen) and blotted to a Biotrace polyvinylidene difluoride membrane (Pall Corporation, Ann Arbor, Mich.). A monoclonal anti-Flag (M2, Sigma) and a donkey horseradish peroxidase-conjugated anti-mouse immunoglobulin antibody were then used (Jackson ImmunoResearch Lab. Inc., West Grove, Pa.). Protein expression was detected with an enhanced Luminol reagent (Renaissance, Boston, Mass.).

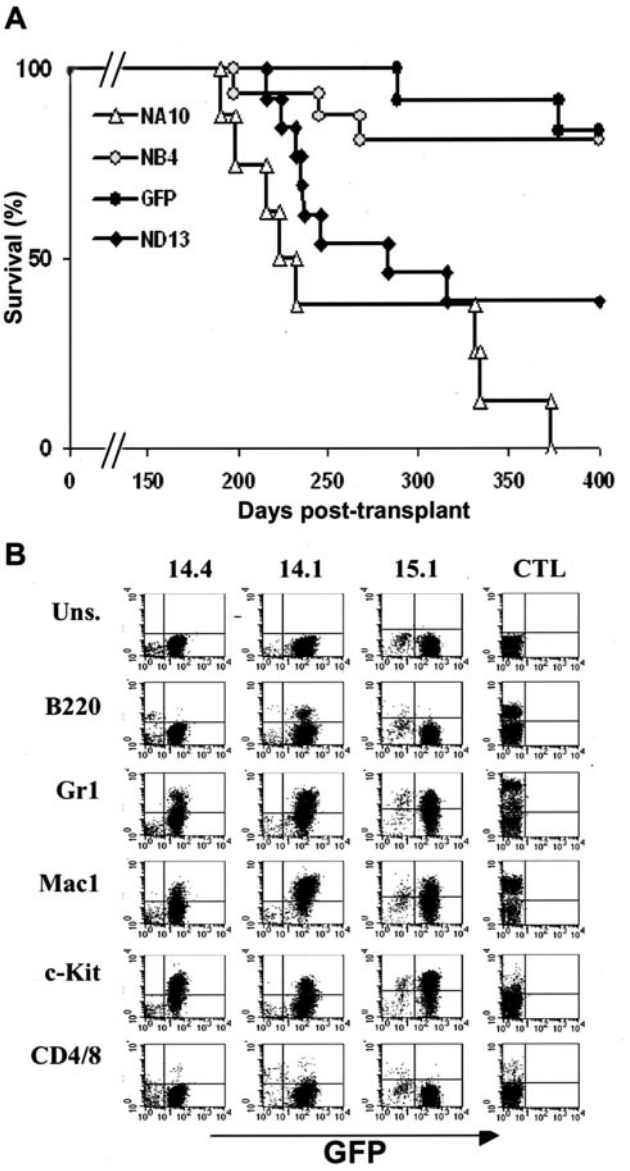

FIG. 2. Novel fusion gene *NUP98-HOXA10* but not *NUP98-HOXB4* induces leukemia in mice. (A) Survival curve of mice transplanted with *NA10* ($n = 8$), *NB4* ($n = 16$), *ND13* ($n = 13$), and GFP ($n = 12$) transduced bone marrow. (B) Immunophenotyping of leukemic cells. Flow cytometry profiles of bone marrow cells for three sick *NA10* recipients and one control mouse (CTL) are shown. Expression of GFP is shown on the *x* axis and that of the cell surface antigens on the *y* axis. Uns, unstained.

Statistical analysis. Data were statistically tested with the *t* test for dependent or independent samples (Microsoft Excel). Differences with P values <0.05 were considered statistically significant.

RESULTS

Redundancy of the ability of *Abd-B*-like *Hox* genes to cause AML as *NUP98* fusion partners. The increasing list of *Abd-B*-like *Hox* genes that have been identified fused to *NUP98* in human AML strongly suggests a functionally equivalent role for this closely related group of genes in leukemia. Interest-

TABLE 2. Hematological characteristics of diseased *NA10* mice

Mice (no.)	Mean day of sacrifice ± SD	Mean no. of white blood cells (10^6/ml) ± SD	Mean no. of red blood cells (10^9/ml) ± SD	Mean spleen wt (g) ± SD	Mean % blasts in marrow ± SD
NA10 (5)	257 ± 70.2	113.5 ± 149	2.7 ± 1.8	0.59 ± 0.27	36 ± 9
Normal B6C3 control (3)		7.1 ± 6.6	6.8 ± 1.4	0.10 ± 0.02	8.2 ± 2.7

ingly, however, this list does not include any of the *Hox* genes belonging to the 10th paralog group, even though *HOXA10* has been reported to be leukemogenic in mouse models. This raises the possibility that *Hox* genes of the 10th paralog group do not possess the properties required to form leukemogenic *NUP98* fusion genes. To clarify this issue, we engineered a novel *NUP98-Abd-B* fusion gene, *NUP98-HOXA10* (*NA10*), and studied the effect of its overexpression in a mouse bone marrow transplantation model. To construct *NA10*, the cDNA portion corresponding to the second exon of *HOXA10*, containing its homeodomain and Pbx-interacting motif, was fused in frame to the *NUP98* portion found in *ND13*, thus recapitulating a typical *NUP98-Hox* fusion gene (Fig. 1). *NA10* cDNA was cloned into the MSCV-IRES-GFP retroviral vector, in which an internal ribosomal entry site allows the cotranslation of the GFP reporter gene and *NA10*.

Expression of *NA10* provided freshly transduced bone marrow cells with a strong growth advantage, as evidenced by the increasing proportion of transduced GFP^+ cells over time in liquid culture (Fig. 1D). This effect was similar to that induced by *ND13* and provided initial support for *HOXA10* as an oncogenic *NUP98* fusion partner. To determine whether the growth advantage could translate into leukemogenic activity in vivo, lethally irradiated mice were reconstituted with *NA10*-transduced bone marrow cells.

NA10-transduced bone marrow cells reconstituted the hematopoietic system of irradiated mice efficiently, with the proportion (Table 1) and expression levels of GFP^+ mononuclear cells (Fig. 1E) similar to that observed for ND13 control-transplanted mice. No significant differences were observed in the number of white blood cells and red blood cells in the *NA10* and control *HOXA10* and GFP recipients 8 weeks post-transplant (Table 1). However, as first detected at 24 weeks, three out of eight healthy-looking *NA10* mice displayed elevated white blood cell numbers (14×10^6 to 35×10^6/ml) due to increased myeloid cells ($Gr1^+$ and/or $Mac1^+$), consistent with a mild myeloproliferative condition, as previously observed in some *ND13* and *NUP98-HOXA9* mice (16, 25).

Recipients transplanted with *NA10*-transduced bone marrow cells had a much reduced survival rate compared to GFP controls but similar to that observed for control *ND13* mice (Fig. 2A), with a median survival of 223 days. Examination of sick *NA10* recipients revealed that they had elevated white blood cell counts (3- to 50-fold above normal), anemia, and splenomegaly (Table 2). An AML phenotype was confirmed by Wright-Giemsa staining of bone marrow cytospins, which revealed a high proportion (≥25%) of blast cells in the marrow of the sick *NA10* recipients (Fig. 3A; Table 2). The myeloid nature of the leukemic cells was confirmed by flow cytometry (Fig. 2B), which revealed that the majority of the leukemic bone marrow cells stained positive for Mac1, Gr1, and c-Kit, although a small degree of phenotypic heterogeneity was observable among different mice. This phenotypic variability likely reflects small differences in the predominant stage of differentiation impairment influenced by the level of NA10 expression or the nature of an extra mutation(s) acquired during leukemia progression (see below).

All leukemias originated from transduced bone marrow cells, as confirmed by GFP expression (Fig. 2B) and Southern blot analysis, which detected the presence of the intact *NA10* provirus (data not shown). Histopathological analysis confirmed significant infiltration of immature myeloid cells in the spleen, liver, kidneys, and occasionally in lungs (data not shown). The leukemia was transplantable, and all secondary recipients injected with as few as 5,000 bone marrow cells also developed AML, with a latency ranging from 31 to 69 days (three primary mice tested). The much-reduced disease latency

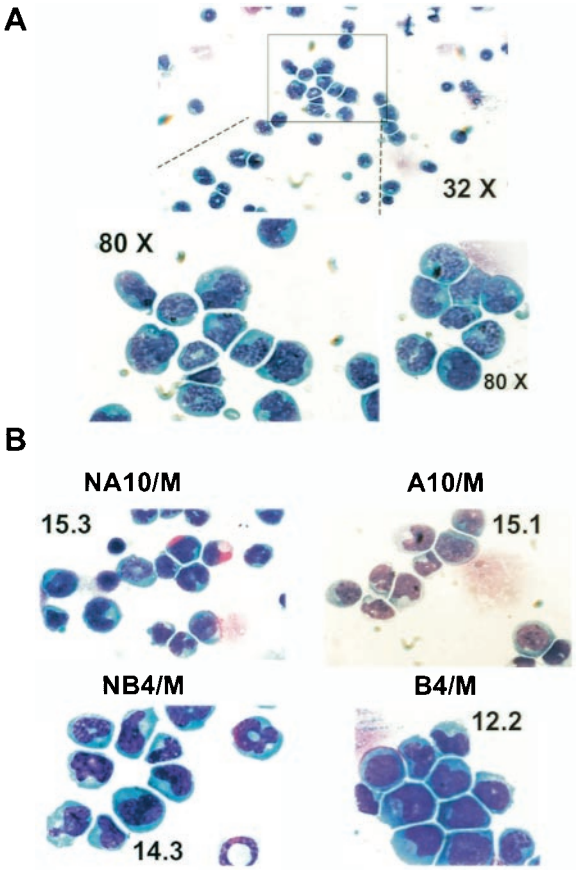

FIG. 3. Wright-Giemsa staining of leukemic bone marrow cells harvested from moribund *NUP98-HOXA10*, *NUP98-Hox/Meis1*, and *HOX/Meis1* mice. Staining of leukemic bone marrow cells harvested from moribund *NA10* mice (A) or from moribund *NUP98-Hox/Meis1* and *Hox/Meis1* mice (B). M, *Meis1*.

seen in the secondary transplants argues that additional mutations had been acquired during the genesis of the leukemias. As previously reported and confirmed here, control *HOXA10* (data not shown) and *ND13* recipients also developed AML over time (25, 37). In contrast to the reported disease kinetics observed between *HOXA9* and *NA9* mice (16), *HOXA10*, associated with a median survival of 151 days, induced AML faster than *NA10*. Together, these results demonstrate that the *Abd-B*-like *HOXA10* gene is a potent oncogenic *NUP98* fusion partner and support a model in which all members of this class of *Hox* genes have overlapping leukemogenic properties.

Fusion of the nonleukemogenic *HOXB4* gene to *NUP98* does not cause AML. The results presented above supported the hypothesis that *Abd-B*-like *Hox* genes have common transforming properties that can be complemented by fusion to *NUP98*. However, it remained possible that any *Hox* gene, regardless of its intrinsic leukemogenic potential, could form an oncogenic fusion gene with *NUP98*. To investigate this, we engineered a fusion between *NUP98* and *HOXB4*, a *Hox* gene previously found to be nonleukemogenic in transplant models and not yet implicated in human leukemia.

In contrast to *NA10*, *NB4* did not induce leukemia even after follow-up of 1 year (Fig. 2A). This lack of leukemogenic activity was not attributable to low-level engraftment or expression levels, as evident from GFP expression in peripheral blood mononuclear cells (Table 1 and Fig. 1E). During that observation period, three *NB4* and two *GFP* recipients died of unknown causes with no evidence of myeloproliferative disorder or hematological abnormalities. Clonogenic progenitor assays and fluorescence-activated cell sorter (FACS) analysis of the marrow of one of the *NB4* mice revealed no apparent perturbation, and all secondary bone marrow transplants remained healthy (>47 weeks). These results indicate that the fusion of *NUP98* to *HOXB4* was without detectable leukemogenic activity and thus suggest that not all *Hox* genes can form leukemogenic *NUP98* fusions.

To discriminate between the possibilities that *HOXB4* could not complement *NUP98* due to lack of intrinsic leukemogenic activity or that only *Abd-B* genes are able to collaborate with *NUP98*, we engineered a fusion between *NUP98* and another *Antennapedia*-like *Hox* gene, *HOXB3*, that on its own has been shown to induce AML. Low viral titers resulted in sufficient numbers of GFP^+ NB3 cells to transplant only two mice; nevertheless, one of two *NB3* recipients developed leukemia, as evidenced by a high white blood cell count (110×10^6/ml), low red blood cell count (5.3×10^9 red blood cells/ml), splenomegaly (0.58 g), and a proportion of blasts of 21% in its marrow at ≈235 days posttransplant, a latency similar to that reported for the intact *HOXB3* gene (31). Together, these data suggest that the ability of *Hox* genes to form leukemogenic fusions with *NUP98* is not restricted to *Abd-B*-like *Hox* genes, but may rather be determined by the intrinsic leukemogenic potential of the *Hox* gene fused.

Redundant ability of *Hox* and *NUP98-Hox* genes to cause AML in collaboration with *Meis1* is independent of their intrinsic leukemogenic potential. The Hox cofactor *Meis1* has been shown to reduce the time of disease onset for several *Hox*-induced leukemias, suggesting redundancy in the ability of *Hox* genes to collaborate with *Meis1*. A second important observation arising from these studies is that the strength of this cooperation seems to correlate with the intensity of the leukemogenic potential of the *Hox* gene involved. In order to clarify these issues, we investigated the potential synergy between *Meis1* and *NA10* (a strong oncogene), *NB3*, and *NB4* (nononcogenic). In addition, to determine whether the synergy between *Meis1* and the fusion genes reflects an intrinsic property inherited from their normal Hox counterparts, both *HOXA10* and *HOXB4* were coexpressed with *Meis1* in vivo.

Bone marrow cells were cotransduced with the various NUP98-Hox-IRES-GFP and Meis1-IRES-YFP retroviruses and injected into irradiated mice. Strikingly, all fusion and *Hox* genes tested collaborated with *Meis1* to induce leukemia, as evidenced by reduced disease latency or, in the case of *NB4* and *HOXB4*, conversion to leukemogenic activity (Fig. 4A and B). Expression of *Meis1* alone did not cause disease, as previously reported (15). Moreover, the median survival seen with *Meis1* in combination with *NA10* or *HOXA10* was much shorter than in combination with *NB3*, *HOXB4*, or *NB4* (Fig. 4B), reflecting the previously documented different leukemogenic potencies of the *Hox* genes by themselves. Moreover, although as single factors *NB4* and *HOXB4* were unable to induce leukemia, both genes were equally capable of inducing the disease with indistinguishable latency in combination with *Meis1* (Fig. 4A and B; Table 3). This ability of nonleukemogenic *Hox* genes to cause AML in collaboration with *Meis1* further supports the redundancy of such collaboration.

The phenotype of the diseased NA10/Meis1 (NA10/M), A10/M, NB4/M, and B4/M mice closely resembled that induced by *NA10* and that previously reported from ND13/M (25), with elevated white blood cell counts, anemia, splenomegaly, and a high percentage of poorly differentiated myeloid blasts in the bone marrow (Fig. 3B; Table 3). The myeloid phenotype of the leukemic cells was confirmed by flow cytometry (predominantly $Gr\text{-}1^+$ $Mac\text{-}1^+$ $B220^-$ $CD4/8^-$, data not shown). Importantly, flow cytometric data from representative mice (Fig. 5A) revealed that leukemic bone marrow cells were positive for coexpression of the transduced genes, as evidenced by the high proportion of GFP/YFP double-positive cells. The origin of the leukemias from cotransduced cells, initially a minority population (as low as 0.4%) in the transplant inocula, was further confirmed by Southern blot analysis, showing the presence of NUP98-Hox and Meis1 or Hox and Meis1 proviruses in primary and secondary leukemic mice (Fig. 5B).

The leukemias caused by NA10/M, A10/M, NB4/M, and B4/M were transplantable, with secondary transplant recipients succumbing to leukemia in 20 to 70 days posttransplant. The frequency of the leukemic initiating cell was estimated by injecting different doses of primary leukemic marrow cells into multiple recipients. All mice from all gene combinations died after receiving as few as 5,000 cells, and the majority of mice receiving 500 cells also succumbed to AML (Table 4), indicating a high frequency of leukemia-initiating cells. Clonal analysis of genomic DNA harvested from the primary and secondary transplants revealed that the majority of the leukemias were of clonal and/or oligoclonal origin, as evidenced by the same banding pattern of equal intensity in the primary and secondary transplants (Fig. 5C). Interestingly, some sick NB4/M and B4/M mice showed a polyclonal bone marrow reconstitution at the time of analysis, as evidenced by the presence of multiple NB4 or B4 proviral bands in the primary

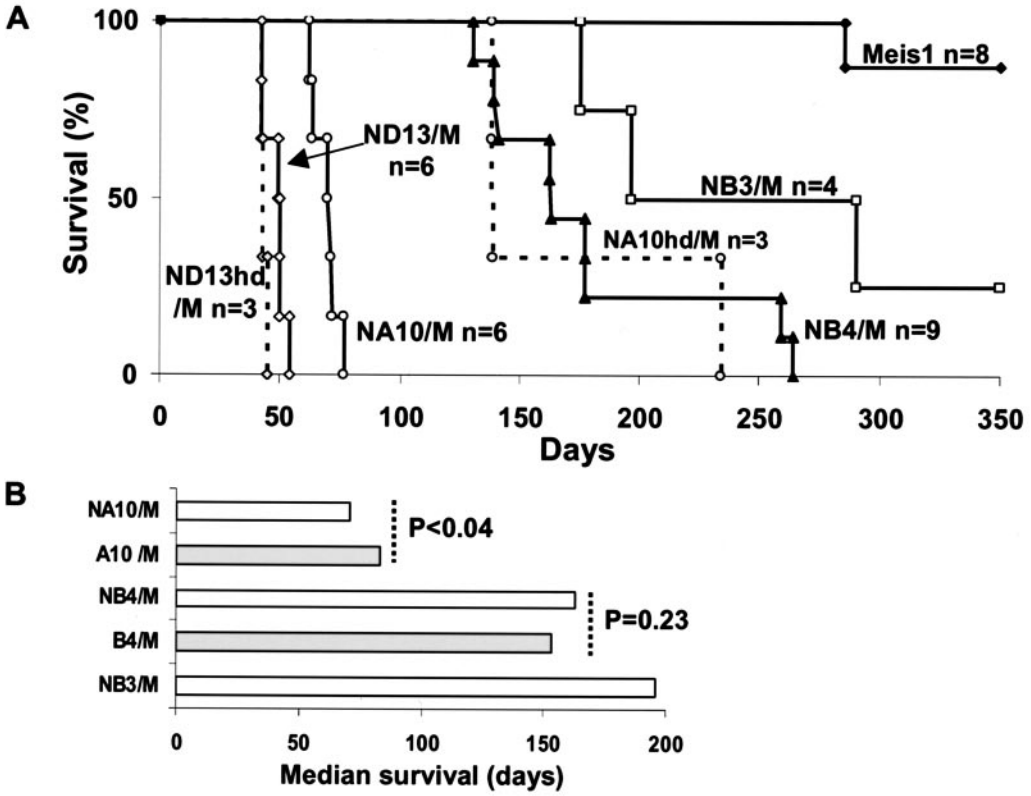

FIG. 4. Coexpression of *Meis1* strongly increases the leukemogenic potential of *NA10* and induces AML with *NB4*, *NB3*, and two native *Hox* genes. (A) Survival curve of mice transplanted with unsorted or enriched cotransduced bone marrow cells (see Materials and Methods for details). (B) The median disease latency for *HOXA10* plus *Meis1* (M) and *NA10* plus *Meis1* is shorter than that for *HOXB4* plus *Meis1*, *NB4* plus *Meis1*, or *NB3* plus *Meis1*. The *t* test *P* values are also shown.

recipients (see NB4/M 10.1 and 15.1 and B4/M 12.1 lanes in Fig. 5C), which is consistent with *HOXB4*'s intrinsic property of enhancing HSC regeneration capacity, a property that seems preserved in *NB4*.

Previous studies of *ND13* revealed that its growth-promoting activity was dependent on an intact homeodomain. These results suggested that the homeodomain might possibly be the most significant contribution of the Hox partner. To determine whether *Meis1* can collaborate with *NUP98* fusion genes containing only the homeodomain encoded by the *Hox* gene, a series of mice were transplanted with bone marrow cells cotransduced with *Meis1* and *ND13hd* or *NA10hd*, in which only the homeodomain (hd) encoded by *HOXD13* (amino acids 265 to 325) or *HOXA10* (amino acids 324 to 385) was fused in frame to *NUP98*. As seen in Fig. 4A, *ND13hd* was as efficient as *ND13* in inducing leukemia in collaboration with *Meis1*, and though following a longer latency, all mice transplanted with NA10hd/Meis1-transduced cells also succumbed to leukemia, indicating that the Hox homeodomain alone, for these genes, is enough for collaboration with *Meis1*.

Strong leukemogenic potential of *Abd-B*-like *Hox* genes correlates with their robust ability to promote cell growth and block differentiation. Based on the results described above, we speculated that the high leukemogenic potential of *Abd-B*-like *Hox* genes might be explained by their strong ability to promote cell proliferation and/or block differentiation.

All three novel fusion genes enhanced the proliferation of transduced bone marrow cells in short-term liquid culture, as

TABLE 3. Hematological characteristics of moribund mice transplanted with bone marrow cells cotransduced with *Meis1* and *Hox* or *NUP98-Hox* genes

Genes transduced (no. of mice)	Mean time of sacrifice (days posttransplant) ± SD	Mean no. of blood cells ± SD		Mean spleen wt (g) ± SD	% Bone marrow cells ± SD	
		White (10^6/ml)	Red (10^9/ml)		Blasts	Myeloblasts
A10/M (3)	106.7 ± 27.7	115.8 ± 89.4	1.95 ± 1.0	0.56 ± 0.16	23.3 ± 5.7	55.7 ± 2.5
NA10/M (3)	69.7 ± 7.5	140 ± 133	5.5 ± 0.9	0.41 ± 0.11	32.0 ± 8.9	50.7 ± 4.7
B4/M (4)	144.3 ± 9.2	131.3 ± 63.7	4.0 ± 2.4	1.0 ± 0.5	28.0 ± 9.1	53.8 ± 3.8
NB4/M (4)	167.8 ± 21.0	65.5 ± 45.0	5.1 ± 2.9	0.7 ± 0.4	20.0 ± 6.0	46.0 ± 8.5
Normal B6C3 (3)		7.1 ± 6.6	6.8 ± 1.4	0.10 ± 0.02	8.2 ± 2.7	10.4 ± 4.1

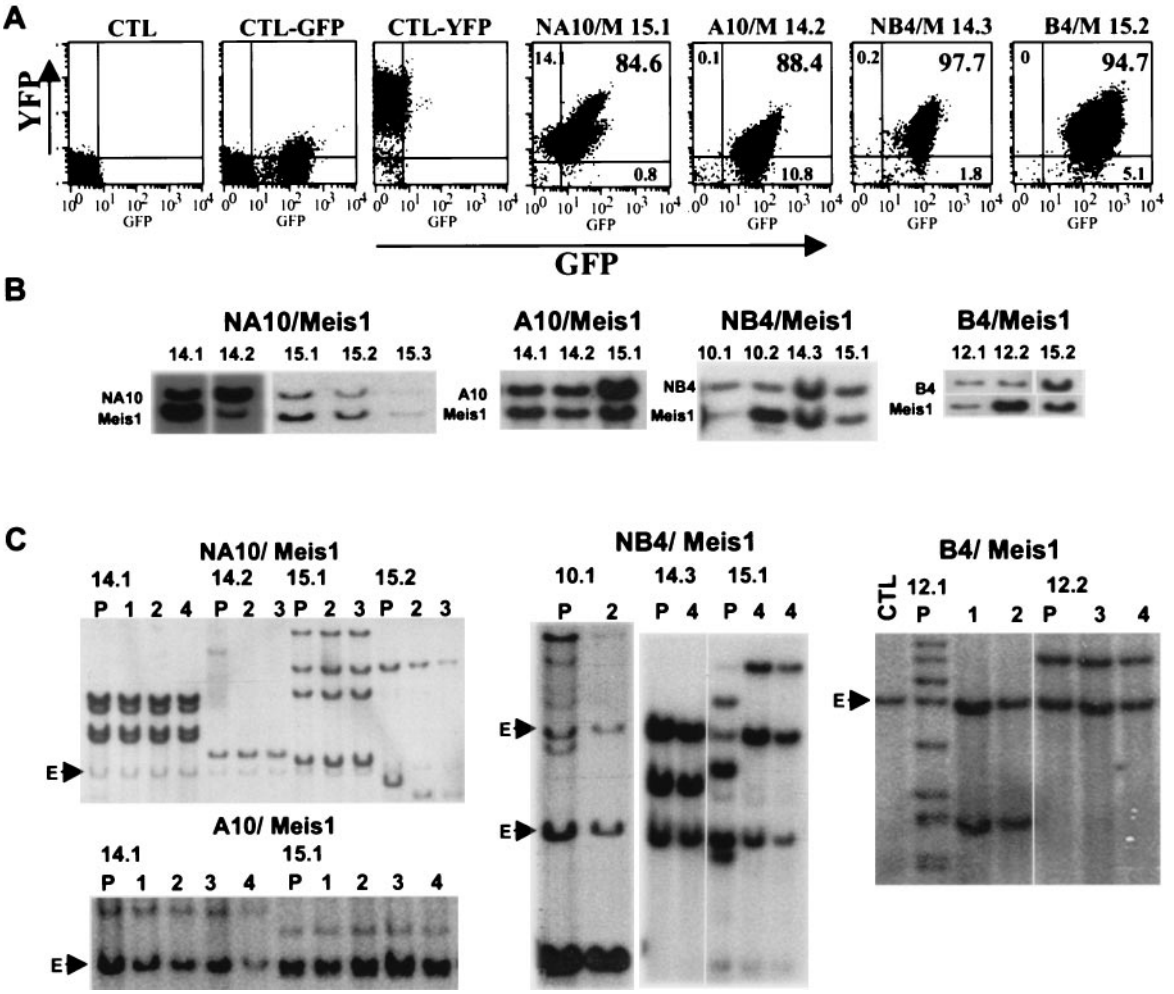

FIG. 5. *NUP98-Hox*/*Meis1*- and *Hox*/*Meis1*-induced myeloid leukemias are of clonal or oligoclonal origin. (A) Analysis of GFP and YFP expression in leukemic bone marrow cells (representative profiles shown). Fresh normal marrow (control, CTL) and GFP- and YFP-transduced control bone marrow cells are also shown. (B) Southern blot analysis from genomic DNA harvested from the leukemic marrow confirmed the presence of the Meis1-IRES-YFP and NUP98-Hox-IRES-GFP provirus in the diseased mice. (C) To estimate the frequency of leukemia-initiating cells and analyze the clonal origin of the leukemias, bone marrow from moribund primary recipients was injected into irradiated secondary recipients at various doses. The lanes loaded with the primary recipient genomic DNA are indicated by P, and the lanes indicated by the numbers 1, 2, 3, and 4 correspond to DNAs of the secondary transplant recipients which received 5×10^5, 5×10^4, 5×10^3, and 500 bone marrow cells, respectively, from the primary recipients. The arrows indicate the locations of endogenous *Hox* or *Meis1* sequences hybridizing to the probes.

evidenced by the increased proportion of GFP^+ cells over time (Fig. 1D and data not shown). The total expansion of GFP^+ cells (i.e., total number of GFP^+ cells after 14 days/input GFP^+ cells) induced by *NA10* was, however, consistently greater (10- to 50-fold higher) than that induced by *NB3* and *NB4* and similar to that obtained for *ND13* (Fig. 6A). Moreover, this differential growth-promoting effect induced by the various fusion genes was also seen in more primitive cells, as shown by day 12 CFU-S. As previously reported, GFP cultures yielded a limited number of day 12 CFU-S cells following 1 week of culture. In contrast, all fusion genes tested produced a prominent increase, an average of 4,700-fold, in the yield of CFU-S-like cells (Fig. 6B). Again, the magnitude of the impact was greater for *NA10*, which produced the highest day 12 CFU-S recovery, matching that observed with *ND13* (Fig. 6B), whereas *NB4* consistently produced the smallest yield in all experiments ($n = 3$), followed by *NB3*.

The impact on bone marrow differentiation was further investigated following a culture period of 3 to 4 weeks. The majority of cells in the GFP^+ control cultures had undergone terminal differentiation, as evidenced by their slow growth and morphology (Fig. 7A; increased cytoplasm with increased granular content). FACS analysis (Fig. 7B) indicated a high level of expression of the c-Kit and IgE receptors, consistent with a mast cell phenotype. Under the same conditions, cultures initiated with *NB4*- and *NB3*-transduced cells consisted of a mixed population of well-differentiated and more primitive myeloid cells (Fig. 7A and B). Most strikingly, *NA10*- and *ND13*-transduced cells yielded cells almost entirely of an immature phenotype, as shown by negative expression of IgE receptors and low levels of c-Kit (Fig. 7B), blast morphology (Fig. 7A), and high proliferation rate. The greater impact on bone marrow differentiation observed for *NA10* was also evidenced in vivo. Phenotypic analysis of peripheral blood of

TABLE 4. Leukemias induced by *NUP98-HOX* fusion genes plus *Meis1* are highly transplantable

Gene combination (no. of mice tested)	% of mice surviving 125 days after transplantation with indicated no. of leukemic bone marrow cells:		Median survival (days) of mice receiving 5,000 leukemic cells
	500	5,000	
NA10/Meis1 (4)	33	0	19.5
A10/Meis1 (4)	25	0	45
ND13/Meis1 (3)	0	0	27
NB4/Meis1 (4)	25	0	46
B4/Meis1 (4)	50	0	69

NA10- and *HOXA10*-transplanted mice before the onset of evident disease also showed evidence of differentiation block, with a significant ($P < 0.03$) decrease of circulating $B220^+$ GFP^+ B cells (Fig. 7C). In contrast, a normal distribution of lymphoid and myeloid GFP^+ cells was observed in *NB4* and *HOXB4* recipients (Fig. 7C).

The different transforming potential and the block in bone marrow differentiation induced by the fusion genes were further confirmed by serial replating of bone marrow clonogenic progenitor cells in methylcellulose. Control GFP^+ cells only produced small clusters of myeloid cells (averaging $1.23 \times 10^3 \pm 5.6 \times 10^2$ cells/colony) in secondary plating, whereas all *NUP98-Hox* genes produced larger secondary granulocyte-macrophage myeloid colonies (ranging from 36×10^3 to 100×10^3 cells/colony). Consistent with the stronger differentiation block induced by *NA10* and *ND13*, bone marrow transduced with these genes formed large granulocyte-macrophage colonies over at least four passages (Fig. 7D). In contrast, *NB4*- or *NB3*-transduced clonogenic progenitors were essentially exhausted by the third replating (Fig. 7D; $n \geq 2$).

DISCUSSION

In this study, we explored whether *Hox* genes have unique or overlapping or redundant roles in leukemogenesis in the context of *NUP98-HOX* fusion genes. Specifically, with novel *NUP98-HOX* fusion gene constructs, we addressed whether *Abd-B*-like *Hox* genes have common intrinsic properties that could make them prime *NUP98* fusion partner candidates, or whether the required properties could extend to other *Hox* members that would suggest a wider overlap in Hox function. Although the engineered *NUP98-Hox* genes originated from two different clusters and four different paralog groups, all four fusion genes tested produced a significant overlap in effects when expressed in bone marrow cells in vitro, reminiscent of the functional redundancy commonly observed for the *Hox* gene family. However, not all *Hox* genes formed strong leukemogenic *NUP98* fusion genes, but under certain circumstances, such as increased *Meis1* expression, all induced myeloid leukemia.

Decisively, the *Abd-B*-like *Hox* genes formed NUP98 chimeric proteins with the greatest impact on proliferation and differentiation in vitro and leukemogenic potency in vivo, which may account for the fact that to date, only *Hox* genes belonging to this group have been found rearranged with

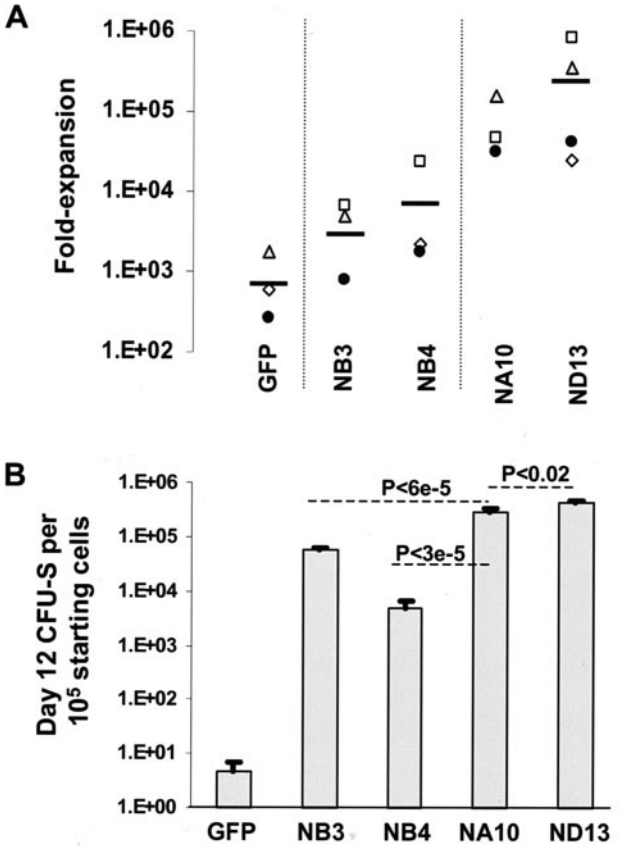

FIG. 6. *NUP98-Hox* genes provide a proliferative advantage to bone marrow cells in vitro. (A) Expression of the fusion genes led to an increased expansion of the transduced bone marrow cells compared to that of GFP^+ control or untransduced cells. Expansion for transduced bone marrow cells in liquid culture over 14 days and the mean (horizontal bar) of at least three experiments are shown. Expansion of cells in *HOXB4*- or *HOXA10*-transduced cells in culture ranged from 2.1×10^3 to 3.6×10^3 and 1.0×10^3 to 3.1×10^3, respectively. (B) Greater yield of day 12 CFU-S colonies after 1 week of culture of 10^5 bone marrow cells transduced with fusions of *NUP98* and *Abd-B*-like *Hox* versus *NB3* and *NB4* (representative experiment shown ± standard deviation [$n = 3$]). The *P* values from the *t* test are also shown ($n = 3$).

NUP98 in human leukemia. Nevertheless, the leukemogenic potentials observed for *NUP98-HOXB4* and *HOXB4* with *Meis1* support a model in which many if not all *Hox* genes may have the not previously appreciated capacity to contribute to leukemic transformation and point to striking overlap in properties.

Intrinsic leukemogenic potential of intact *Hox* genes correlates with their ability to induce AML as *NUP98* fusion partners. The novel *NA10* fusion gene induced AML with kinetics similar to that of *ND13*, indicating that the reported leukemogenic potential of *HOXA10* (37) is retained in the *NA10* fusion. Our data further imply that the ability to form leukemogenic *NUP98-Hox* fusion genes is not restricted to the already identified *Hox* genes, but rather seems to be a redundant property of *Abd-B*-like *Hox* genes. These results are consistent with reported functional identity between paralogous as well as nonparalogous *Abd-B*-related *Hox* genes (10, 38, 39). *Abd-B*-like *Hox* genes are derived from a common ancestor, and

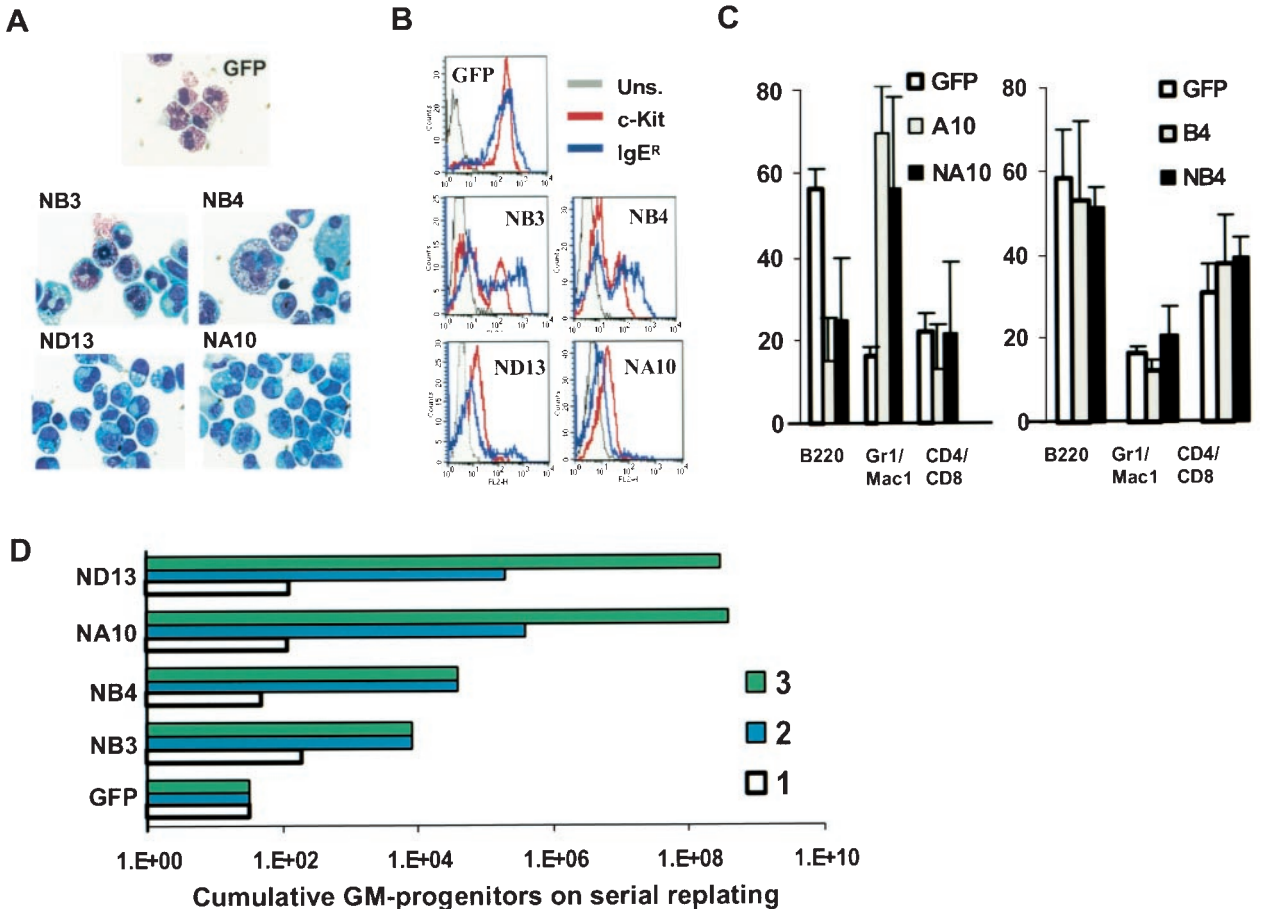

FIG. 7. *NUP98–Abd-B*-like *Hox* fusion genes have a greater transforming potential in vitro. (A) Wright-Giemsa staining of transduced bone marrow cells grown for 3 weeks in liquid culture. Magnification, ×80. Note the reduced number of mature granulocytic cells for *NA10*- and *ND13*-transduced cells compared to more differentiated GFP^+ control cells (representative experiment shown [$n = 2$]). (B) Immunophenotyping of cells after liquid culture. Unstained cells are in grey; IgE receptor (IgE^R) and c-Kit are shown in blue and red, respectively. (C) Lineage distribution of GFP^+ cells in transplanted mice. Mean distribution for the cohorts ± standard deviation. Analysis was done 24 weeks posttransplant for *GFP* ($n = 3$), *A10* ($n = 2$), and *NA10* ($n = 4$) mice and 30 weeks posttransplant for *B4* ($n = 3$) and *NB4* ($n = 3$). (D) Total number of granulocyte-monocyte progenitors in serial replating. Cumulative yield is shown for the initial plating of 750 transduced bone marrow cells (representative experiment shown [$n \geq 2$]).

therefore their homeodomains are closely related, which may indicate that their redundant function in leukemogenesis is mediated through the regulation of a similar or overlapping set of genes and/or pathways that control hematopoietic cell growth and differentiation.

Our results showing strong leukemogenic potential for *NA10* but not for *NB4* suggest that the intrinsic leukemogenicity of *NUP98* fusions is determined primarily by the *Hox* fusion partner. Moreover, such intrinsic leukemogenicity appears not to be restricted to *Abd-B*-like *Hox* genes, as suggested by leukemia arising in an *NB3* recipient. This differential ability of *NB3* and *NB4* to cause leukemia is consistent with the specific effects of their native counterparts (30, 31) and further demonstrates that closely related *Hox* genes are not completely functionally equivalent (26, 40).

AML is characterized by the uncontrolled proliferation of myeloid cells that accumulate at different stages where their further differentiation is blocked. The high correlation observed between the leukemogenic potential of the native and *NUP98-Hox* genes and their ability to increase self-renewal and block differentiation argues that the latter characteristics determine the Hox leukemogenic potential. Thus, the intrinsic ability of *ND13* and *NA10* and that reported for *NA9* (7) to sustain increased self-renewal and strongly block bone marrow differentiation appear to be a unifying property that determines their potent leukemogenicity.

Redundant ability of *Hox* genes to collaborate with *Meis1* to induce AML. Despite considerable differences in the function and structure of the native and *Hox*-fused genes studied in this work, they were all able, though with different latencies, to cause AML in collaboration with *Meis1*. Importantly, our data demonstrating the capacity of *NB4* and *B4* to cause AML with *Meis1* indicate that even *Hox* genes that do not cause leukemia on their own possess the redundant ability of becoming leukemogenic in the presence of this, and possibly other, cofactors.

Work from Calvo et al. showing that *Meis1* may further block differentiation of myeloid progenitors overexpressing *HOXA9* (5), coupled to the ability of *HOXB4* to transform fibroblasts in vitro (17), could in part explain the leukemogenic activity of *HOXB4* and *NB4* in collaboration with *Meis1*. Meis1

has been shown in myeloid cells to be associated with ternary complexes containing Pbx and Hox proteins (34), in which Meis1 directly interacts with Hox proteins from paralog groups 9 to 13, or indirectly associates with paralogs 1 to 8 through Pbx (33). NA10 and NA9 both retain the Pbx interacting motifs and therefore, Meis1 may indirectly interact with these proteins through the formation of trimer complexes containing Pbx, as suggested for A9-mediated transformation of myeloid progenitors (32). Arguing against the likelihood that such direct interactions are the key to leukemic transformation, several of the fusions tested (NB3, NB4, ND13, ND13hd, and NA10hd) lacked both known Pbx1- and Meis1-interacting motifs. Nevertheless, the longer latency observed for NA10hd/Meis1 (lacking a Pbx-interacting motif) compared to NA10/Meis1 (containing a Pbx-interacting motif) suggests that interaction with Pbx proteins, though dispensable, may accelerate disease development.

How *Meis1* accelerates the onset of Hox-mediated AML is still unclear. Whether *Meis1* functions to increase the strength of the transcriptional activity of *Hox* genes on Hox-regulated promoters or works through different target genes needs to be resolved. Several non-mutually exclusive explanations could be proposed. First, in view of work from Saleh et al. showing that under some circumstances Pbx1 can repress *Hox* gene-mediated transcription by interacting with multiple histone deacetylases (28) and a recent report showing increased ability of *HOXB4* to enhance self-renewal of HSC when Pbx1 was downregulated (18), one could propose that Meis1, known to form strong heterodimers with Pbx1, might bind and titrate off Pbx proteins. Another possibility is that the exogenous Meis1 physically interacts with endogenously expressed Hox proteins also implicated in leukemia, such as Hoxa7 and Hoxa9 (7, 25). *Meis1* might also directly bind endogenous Hox-responsive genes and/or different genes, through Hox-independent activity (23). This possibility is supported by data indicating that Homothorax, a *Drosophila* Meis1 orthologue, possesses transcriptional activation activity of its own (12).

The *NUP98-Hox* fusion gene transformation models exploited here should now prove useful to dissect the molecular and cellular pathways involved in the redundant and differential leukemogenic potential of *Hox* genes and to provide new strategies to elucidate the role of collaborating genes.

APPENDIX

Supplementary materials. Primer sequences to amplify indicated Hox regions for NUP98-HOX fusion constructs were as follows: NUP98 portion, forward, 5-AGTCGGATCCTTTAACAAATCATTTGGAACACCCTT-3, and reverse, 5-TTTAAGAATTCTACTGGGGCCTGGGG-3; HOXB4 Exon2, forward, 5-CCCAGGAATTCGTAAACCCCAATTACGCC-3, and reverse, 5-ACATAAAAATGCGGCCGCTAGAGCGCGCGGGGGCC-3; HOXB3 Exon4, forward, 5-ATACCCGAATTCGCAGAGGGCTGTGGT-3, and reverse, 5-CCCATCCCCTAATCCTCGAGCGC-3; HOXA10 Exon2, forward, 5-TTATCCGAATTCGGCAATTCCAAAGGTGAA-3, and reverse, 5-TTCAATAATGCGGCCGCTCATCAGGAAAAATT-3; HOXA10 homeodomain, homeobox (hd) A10hd; forward, 5-ACTGATGAATTCAAGAGTGGTCGGAAGAAGCGC-3; reverse, 5-ATAAAAATGCGGCCGCTCACATTTTCTTCAGTTT-3; HOXD13 homeodomain, forward, 5-ACTGATGAATTCCGAAGAGGGAGGAAGAAG-3, and reverse, 5-ACTGATCTCGAGTCATTTCTTGTCCTTCACTCTTCGGT-3.

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