J. Biochem. **141,** 719–727 (2007)
doi:10.1093/jb/mvm073

Co-repressor SMRT and Class II Histone Deacetylases Promote Bach2 Nuclear Retention and Formation of Nuclear Foci that are Responsible for Local Transcriptional Repression

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Received February 23, 2007; accepted February 26, 2007; published online March 23, 2007

Bach2 is a member of the BTB-basic region leucine zipper factor family and represses transcription activity directed by the TPA response element, the Maf recognition element (MARE) and the antioxidant-responsive element. Recently, it was reported that upon oxidative stress Bach2 forms nuclear foci surrounding the promyelocytic leukaemia (PML) bodies and specifically represses the transcription around the PML bodies. Here we report that expression of the silencing mediator of retinoid and thyroid receptor (SMRT) and histone deacetylase4 (HDAC4) enhances the formation of the Bach2 foci in the nuclear matrix. SMRT mediates the HDAC4 binding to Bach2, and HDAC4 facilitates the retention of Bach2 in the foci. Scratch transcription labelling and 3D-reconstruction from the confocal images demonstrated that transcription is suppressed in and around the Bach2 foci. Indeed, Bach2 bound MARE and repressed the expression from the chromosomally integrated MARE-driven reporter gene when co-expressed with SMRT and HDAC4. Our observations suggest that both SMRT and HDAC4 play an important role in nuclear retention and the Bach2 focus formation in the mammalian cell nucleus, which may contribute to the local transcription repression.

Key words: Bach2, HDAC4, nuclear domain, SMRT, transcription repression.

Abbreviations: CLS, cytoplasmic localization signal; ChIP, chromatin-immunoprecipitation; HDAC, histone deacetylase; LMB, leptomycin B; MAD body, matrix-associated deacetylase body; MARE, Maf recognition element; NES, nuclear export signal; PML, promyelocytic leukaemia; SMRT, silencing mediator of retinoid and thyroid receptor.

Recent advance in the imaging techniques have revealed that the mammalian cell nucleus contains distinct classes of subnuclear bodies, including nucleoli (*1*), nuclear speckles (*2*), Cajal bodies (*3*) and promyelocytic leukaemia (PML) bodies (*4*). These nuclear bodies may act as the special domains to expedite the execution of particular nuclear functions such as ribosome biosynthesis, splicing and transcription, by assembling the related factors. A nuclear focus containing Bach2 is one of such nuclear bodies, which is probably involved in the transcriptional repression. Bach2 was originally identified as a protein that interacts with MafK by yeast two-hybrid screening. It possesses a b-Zip domain in the C-terminal region, thus belonging to the CNC-type b-Zip transcription factor family. Unlike other members of this family, however, it has a characteristic BTB domain in the N-terminus. Bach2/MafK heterodimer specifically represses transcription activity directed by the Maf recognition element (MARE) and the antioxidant-responsive element (ARE) (*5*, *6*). Recently, analysis using Bach2 knock-out mice revealed that Bach2 plays an important role in both the immunoglobulin class switch and somatic hypermutation (*7*).

Importantly, Bach2 function is spatially regulated in cells by the nuclear transport factors. The nuclear export factor CRM1 directs its cytoplasmic localization in the normal culture conditions. However, Bach2 is translocated from the cytoplasm into the nucleus upon oxidative stress (*5*), and forms a characteristic nuclear body named the Bach2 focus (*8*, *9*). The Bach2 foci selectively envelope the PML bodies and repress the transcription activity associated with the nuclear bodies (*8*). The BTB domain is essential for the formation of

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the Bach2 foci (*8*). However, the molecular basis for the formation of the Bach2 foci still remains unclear.

Recent studies have shown that the BTB domains of PLZF or Bcl6 form a dimer to make the interface for interacting with the co-repressor SMRT (silencing mediator of retinoid and thyroid receptor) or its related protein N-CoR (*10*, *11*). The overall homology of BTB domains among these proteins is not very high, but several important amino acid residues involved in the binding to SMRT are fairly conserved in Bach2 (Fig. 1A). Indeed, it has been already reported that Bach2 interacts with SMRT in the yeast two-hybrid assay (*12*). It is therefore possible that SMRT/N-CoR contributes to the formation of the Bach2 focus, although this idea has not been experimentally examined.

Histone deacetylases (HDACs) are an important class of enzymes that repress the transcription activity by removing the acetyl group from core histones, which is a marker for transcriptionally active chromatin. Several DNA-binding transcriptional repressors have been shown to co-localize with the co-repressors and/or HDACs. Indeed, HDACs and SMRT have been shown to accumulate in the subnuclear domains called matrix-associated deacetylase (MAD) bodies (*13*, *14*). It is therefore possible that not only co-repressor but also HDACs contribute to the formation of the nuclear bodies responsible for the local transcription. In the present study, we demonstrate that both SMRT and class IIa HDACs are involved in the formation of the Bach2 focus, which might function as a centre for transcriptional repression.

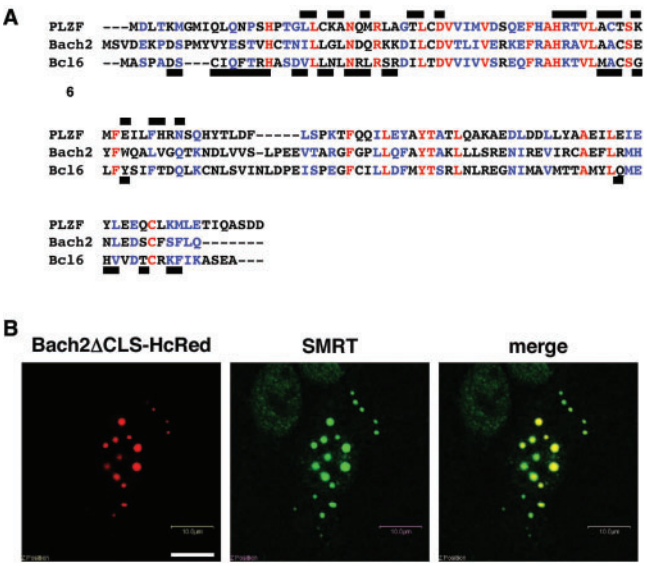

Fig. 1. **Comparison of BTB domains among three BTB-type repressors.** (A) N-terminal 130 amino acids in BTB domains of Bach2, Bcl6 and PLZF were aligned with a clustalw software. Upper and under lines represent important residues for interaction with SMRT (*11*, *19*). (B) The Bach2 focus contains endogenous SMRT. MCF7 cells were transfected with Bach2ΔCLS-HcRed and EGFP-HDAC4ΔNES expression vectors. Endogenous SMRT was stained with an anti-SMRT antibody. A scale bar in the left panel indicates 10 μm.

MATERIALS AND METHODS

Plasmids—pCMV/Bach2 and pcDNA/HDAC4-FLAG were described previously (*6*, *15*). An expression plasmid for HA-SMRT$^{1205–1668}$ was constructed as follows. The cDNA corresponding to residues 1205–1668 of SMRT was isolated as a *Xho*I fragment from pACT-SMRT, and inserted into the *Xho*I site of pACT2 (Clontech), resulting in pACT2-HA-SMRT$^{1205–1668}$. A eukaryotic expression plasmid pEGFP-C1 (Clontech) was digested with *Nhe*I and *Bgl*II to remove the green fluorescent protein-coding region and circularized again, resulting in pCMVKM. A *Bgl*II fragment from pACT2-HA-SMRT was inserted into the *Bam*HI site of pCMVKM to generate pCMV-HA-SMRT$^{1205–1668}$. An *Eco*RI fragment from pcDNA/HDAC4-FLAG was inserted into the *Eco*RI site of pGBT9 (Clontech), resulting in pGBT-HDAC4. pACT2-HDAC4 was constructed as follows. pACT2 was digested with *Nde*I, blunted with the Klenow fragment, and circularized again to shift the reading frame in the MCS site, for constructing pACT2-c2. pACT2-HDAC4 was constructed by inserting an *Eco*RI fragment from pcDNA/HDAC4-FLAG into the *Eco*RI site of pACT-c2. pRBGP2-Hygro^{r} was constructed as follows. The hygromycin resistance gene cassette was isolated as the *Hin*dIII/*Nsi*I fragment from pCMV-lacI (STRATAGENE), and inserted into *Hin*dIII and *Sma*I sites of pBS-SK (STRATAGENE), resulting in pBS-Hygro^{r}. The *Sal*I/*Bam*HI fragment was inserted into *Sal*I/*Bam*HI sites of pRBGP2 (*16*), for constructing pRBGP2-Hygro^{r}.

Transfection and Immunofluorescence Microscopy—Human cell lines HEK293T and GM02063 were maintained in DMEM supplemented with 10% fetal bovine serum and seeded on glass coverslips in 6-well dishes 24 h before transfection. The cells were transfected with the expression plasmids by the calcium phosphate precipitation or a Fu-GENE6 reagent (Roche). Cells were fixed with 4% paraformaldehyde, and then were permeabilized with PBS containing 0.1% SDS and 0.5% Triton X-100/1 for 5 min. After the cells had been incubated for 30 min at 37°C, proteins were visualized by using various combinations of rat anti-HA mAb (diluted 1:200, 3F10, Rosch), mouse anti-FLAG mAb (diluted 1:200, M2, Sigma) and rabbit anti-Bach2 (diluted 1:500; (*6*)) as the primary antibodies, and Alexa488- or Alexa594-conjugated secondary antibodies (Molecular Probes). Nuclei were stained with 10 μM Hoechst 33342. Images were collected using a DeltaVision system (Applied Precision) with a Olympus IX70 fluorescence microscope equipped with an UPlan Apo 100 lens.

Nuclear Matrix Fractionation—The transfected cells were harvested and then cellular proteins were fractionated as below. After being washed with PBS, cells were lysed in cytoskeleton (CSK) buffer (10 mM pipes, pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) supplemented with 0.5% (v/v) Triton X-100, 1 mM DTT, 1 mM EGTA, 1 mM PMSF, a complete protease inhibitor mixture (Roche) at 4°C for 10 min. The cytoskeletal frameworks were separated from soluble proteins by centrifugation at 7500 × *g* for 3 min (fraction 1). The pellet was washed twice with a solution containing 250 mM sucrose and 5 mM $MgCl_2$ (fraction 2) and

resuspended in 25 mM Tris–HCl, pH 7.4/250 mM sucrose/5 mM $MgCl_2$/1 mM PMSF. Chromatin was solubilized by DNA digestion with 1 mg/ml of DNase I at 30°C for 1 h. The sample was centrifuged at $7500 \times g$ for 3 min (fraction 3). The pellet was washed three times with a low-salt buffer (10 mM Tris–HCl, pH 7.4/0.2 mM $MgCl_2$/1 mM PMSF) (fraction 4), extracted consecutively with the low-salt buffer containing an increasing concentration of NaCl (0.3, 0.5 and 2.0 M) for 15 min and centrifuged at $20,000 \times g$ for 15 min (fractions 5, 6 and 7, respectively). The high-salt pellet was finally extracted with the low-salt buffer containing 1% (v/v) Triton X-100 for 15 min and centrifuged at $20,000 \times g$ for 15 min (fraction 8). The remaining pellet was washed twice with the low-salt buffer and solubilized in SDS–PAGE loading buffer (fraction 9). The supernatants were separated with SDS–PAGE, then immunoblotting was performed with antibodies for Bach2 (*6*), FLAG (M2, Sigma) and HA (3F10, Rosch), MafK (*17*) and Lamin B (Goat polyclonal, Santa Cruz).

Yeast Two-hybrid Analysis—To examine protein interactions in yeast cells, AH109 strain was transformed with various combinations of yeast two-hybrid plasmids. Transformants were diluted in water and spotted onto His^+ and His^- media to test for activation of the GAL4-dependent HIS3 reporter gene.

In Vivo Transcription Labelling and Image Acquisition—Scratch transcription labelling was performed as described previously (*8*). Briefly, BrUTP (Sigma) was added to the culture medium at a final concentration of 1 mM. Cells were scratched with a 27G hypodermic needle, and incubated for 5 min at 37°C before fixation with 4% paraformaldehyde in PBS. Samples were examined with a ZEISS LSM510 confocal laser scanning microscope (Carl Zeiss). For the 3D reconstruction of the confocal sections, Amira (TGS) was used.

Establishment of RBGP2 Cell Lines and Reporter Assay—pRBGP2-$Hygro^r$ was linearized with *Bgl*I digestion, and introduced into HEK293T cells. Then the cell clones were selected with hygromycin and luciferase activity, and established as RBGP2 cell lines. RBGP2 cells were seeded in 24-well dishes 24 h before transfection. The cells were transfected with effector plasmids as described previously. The transcription activity was analysed with a Luciferase Assay System (Promega) according to the manufacturer's protocol. Luciferase activities were measured with a Luminescencer-JNR (ATTO, AB-2100). Three independent experiments, each carried out in duplicate, were performed, and the results were averaged and diagrammed with the SE values.

Chromatin-immunoprecipitation (ChIP) Assay—Chromatin fixation and purification procedures were as described previously (*18*). In brief, cell suspensions (1×10^8) were prepared as described and cells were fixed by adding 11% formaldehyde for 5 min at room temperature. Cells were then sonicated to prepare chromatin suspensions of ~900 bp DNA length. Immunoprecipitation was carried out using an anti-FLAG polyclonal antibody (Sigma), an anti-HA monoclonal (3F10, Rosch) or an anti-small Maf polyclonal antibody (*17*) as described previously (*18*). PCR was carried out using LA-Taq DNA polymerase (TAKARA). Amounts of products were measured using a NIH image software, and MARE PCR products were normalized with the amounts of the TK PCR products. The primers used in the PCR were as follows: MARE-Fwd, 5′-TGGTGGTGCAAATCAAAGAACTGC-3; MARE-Rev, 5′-CCAACAGTACCGGAATGCCAAGCT-3′; TK-Fwd, 5′-GGCGGTGTCCCCGGAAGAAAT-3′; TK-Rev, 5′-CGGCACGCTGTTGACGCTGTT-3′.

RESULTS

A Role of SMRT and HDAC4 in Bach2 Focus Formation—The BTB domain in Bach2 has been shown to be required for the Bach2 focus formation (*8*). The Bach2 BTB domain shows 41 and 30% identities, respectively, with those of Bcl6 and PLZF, transcriptional repressors (Fig. 1A). PLZF and Bcl6 are associated with the co-repressor SMRT, and localized in characteristic nuclear domains (*11*, *19–25*). Since the amino acid residues of Bcl6 and PLZF likely important for the interaction with SMRT are highly conserved (*10*, *11*), it seemed possible that SMRT is also co-localized in the Bach2 containing nuclear bodies. To test this possibility, we expressed a fluorescent protein (HcRed)-fused Bach2 protein lacking the C-terminal domain that is required for its cytoplasmic localization signal (CLS) in MCF7 cells, in which endogenous SMRT was expressed at a relatively high level. As shown in Fig. 1B, the Bach2 derivative that is constitutively localized in the nucleus formed nuclear foci, and endogenous SMRT was co-localized with the Bach2 foci.

SMRT is a large protein consisting of 2507 amino acid residues. The domain functions have been analysed in detail (*14*, *26*, *27*), and the repression domain 3 (RD3, 1013–1487 amino acid residues) of SMRT was shown to be sufficient for supporting the formation of the nuclear bodies (*14*). On the other hand, the region of SMRT necessary for the association with Bach2 has been mapped between 1205–1668 amino acids residues (*12*). This region contains silencing domains, SRD-1 and SRD-2, and is also enough to associate with Bcl6 (*28*). Thus we constructed the plasmid for expression of HA-tagged $SMRT^{1205–1668}$ and introduced it into HEK293T cells. As shown in Fig. 2A, Bach2 alone was localized in the cytoplasm of HEK293T cells (Fig. 2A, a), while $SMRT^{1205–1668}$ was efficiently distributed in characteristic subnuclear domains (Fig. 2A, b). When both Bach2 and $SMRT^{1205–1668}$ were simultaneously expressed, the translocation of a significant subpopulation of Bach2 into the SMRT-containing nuclear foci was observed, although the majority of Bach2 was still localized in the cytoplasm (Fig. 2B, d and e).

It has been shown that the co-repressor SMRT directly interacts with class IIa HDACs including HDAC4, which shuttle between the nucleus and the cytoplasm (*29–33*). SMRT is shown to co-localize with these HDACs to form MAD bodies (*13*, *14*). Since $SMRT^{1205–1668}$ is overlapped with the region important for the association with the HDAC4 and HDAC5 (*33*), we tested the possibility that HDAC4 affects the Bach2 focus formation. HDAC4 was localized in the cytoplasm, when expressed in

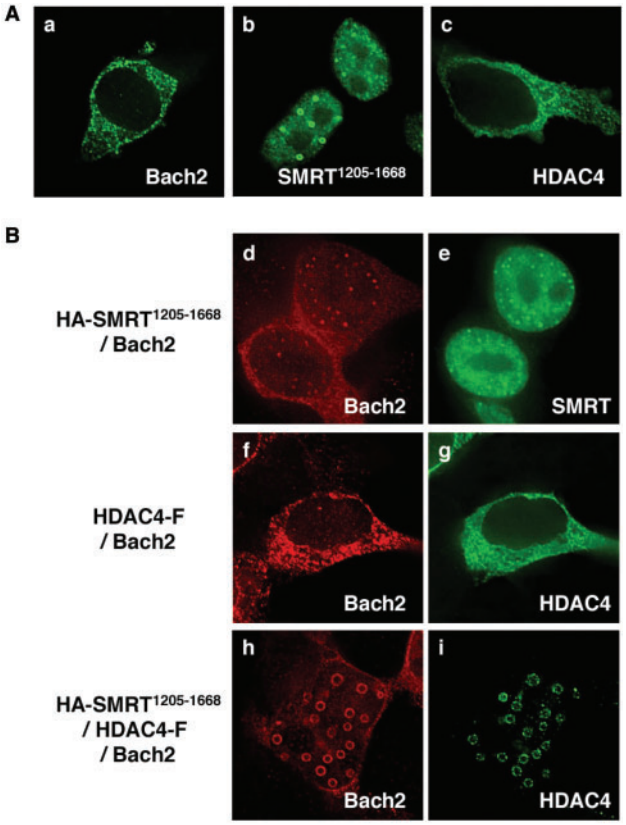

Fig. 2. **Both SMRT and HDAC4 promote Bach2 focus formation.** (A) Subcellular localization of Bach2, HA-SMRT$^{1205–1668}$ and HDAC4-FLAG. Bach2 (a), HA-SMRT$^{1205–1668}$ (b) or HDAC4-FLAG (c) expression vector was introduced into HEK293T cells. (B) Bach2 focus formation in cells overexpressing SMRT and/or HDAC4 cells. Each combination among Bach2, HA-SMRT$^{1205–1668}$ and HDAC-FLAG was co-expressed in HEK293T cells, and Bach2 (d, f and h), HA-SMRT$^{1205–1668}$ (e) and HDAC4-FLAG (g and i) were stained with anti-Bach2, anti-HA (3F10) and anti-FLAG (M2) antibodies, respectively.

HEK293T cells (Fig. 2A, c), and the HDAC4 expression caused no increase in the Bach2 nuclear localization and focus formation (Fig. 2B, f and g). Surprisingly, however, simultaneous expression of SMRT$^{1205–1668}$ and HDAC4 induced nuclear translocation of Bach2 and accelerated the formation of the Bach2 focus (Fig. 2B, h). Although HDAC4 was normally distributed in the cytoplasm, it re-localized to the nuclear bodies when co-expressed with SMRT$^{1205–1668}$ (Fig. 2B, i). Other members of class IIa HDACs, such as HDAC5 and HDAC7, also showed co-localization with Bach2 and SMRT in the nuclear foci (data not shown). These results suggest that SMRT plays a major role in Bach2 focus formation whereas HDAC4 and its related HDACs facilitate accumulation of Bach2 into the nuclear bodies presumably by promoting Bach2 nuclear localization.

Effect of HDAC4 on the Nuclear Transport of Bach2—It seems likely that nuclear import of the cytoplasmic Bach2 protein is prerequisite for the focus formation. Both Bach2 and HDAC4 are regulated by Crm1-dependent nuclear export, which can be inhibited by a specific export inhibitor leptomycin B (LMB) (*5*, *34*, *35*). We therefore tested whether LMB treatment enhances the focus formation (Fig. 3). Inhibition of protein nuclear export by LMB caused rapid nuclear accumulation of Bach2 (Fig. 3A) and HDAC4 (data not shown). In the absence of SMRT, however, nuclear accumulated Bach2 did not form the apparent nuclear foci (Fig. 3A). This result seems inconsistent with our observation that Bach2 mutant lacking CLS formed nuclear foci in MCF7 cells (Fig. 1B). This is probably due to the low level of endogenous SMRT expression in HEK293T cells (*14*), because most Bach2 protein in the nucleus was re-localized into the foci, when SMRT is co-expressed. Bach2 and SMRT were co-localized in the nuclear foci, but not in the cytoplasm (Fig. 3B). The nuclear accumulation of Bach2 by the treatment with LMB caused the formation of larger foci in the presence of SMRT (Fig. 3B, LMB+). Thus, the Bach2 nuclear localization prompted the focus formation only in the presence of SMRT.

We next examined the possibility that HDAC4 accelerates nuclear import of Bach2 by some mechanisms, *e.g*. competition for the nuclear export factors with Bach2. To determine the nuclear import rate of Bach2, we monitored a population of cells with the nuclear localization of Bach2 after the addition of LMB to the cells expressing SMRT and/or HDAC4. Cell population with various Bach2 localization (C > N, C = N and C < N) was counted using the criteria shown in Fig. 3A. As shown in Fig. 3C, the increase in the cells with Bach2 nuclear localization was observed in a manner dependent on the time of LMB treatment, and the Bach2 nuclear localization was detected in >90% of cells within 60 min. However, the Bach2 nuclear import rate was not accelerated by HDAC4. These results deny the possibility that HDAC4 facilitates nuclear import of Bach2. Even in the presence of HDAC4, Bach2 focus formation was not detected during LMB treatment unless SMRT was co-expressed.

SMRT Mediates Interaction between Bach2 and HDAC4—Bcl6 and PLZF have been shown to directly interact with class IIa HDACs (*36*, *37*). To see whether Bach2 can also directly associate with HDAC4, we tried to detect their interaction by the yeast two-hybrid analysis. As shown in Fig. 4A, yeast cells containing AD-SMRT and DBD (DNA-binding domain)-Bach2 or DBD-HDAC4 formed colonies in the His (−) plates, indicating that the interaction between SMRT$^{1205–1668}$ and Bach2 or HDAC4 occurs in yeast cells. However, the interaction between Bach2 and HDAC4 was not detected (Fig. 4B). Furthermore, we found that the rate of nuclear import measured by the inhibition of nuclear export by LMB was also different between Bach2 and HDAC4. HDAC4 was transported more rapidly than Bach2 into the nucleus 15 min after the LMB challenge (Fig. 4C), suggesting that Bach2 and HDAC4 behave differently in the absence of SMRT. These results indicate that SMRT mediates the interaction between Bach2 and HDAC4 in the focus.

Subnuclear Localization of the Bach2 Focus—The co-repressor complex containing a population of HDACs has been shown to be associated with the internal

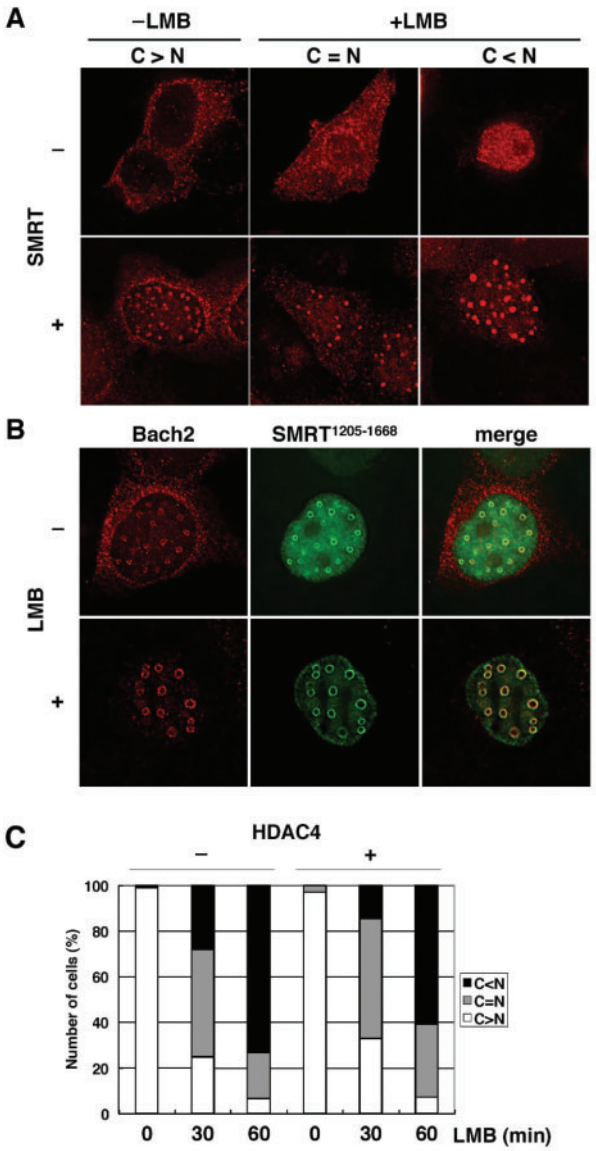

Fig. 3. **Roles of SMRT and HDAC4 in Bach2 nuclear localization and the focus formation.** (A) SMRT is necessary for Bach2 focus formation. Bach2 with or without HA-SMRT$^{1205–1668}$ were expressed in HEK293T cells. Cells were treated with 10 ng/ml LMB for 30–60 min, and stained with an anti-Bach2 antibody. (B) LMB enhances Bach2 focus formation. HEK293T cells expressing Bach2 and HA-SMRT$^{1205–1668}$ were treated with 10 ng/ml LMB for 60 min. Cells were then immunostained with anti-Bach2 and anti-HA (3F10) antibodies. (C) Bach2 nuclear import assay. HEK293T cells transfected with the Bach2 expression vector in the presence or absence of HDAC4-FLAG was treated with 10 ng/ml LMB for 0, 30 and 60 min, and immunostained with anti-Bach2 and anti-FLAG (M2) antibodies. Over 100 cells were divided into three patterns of distribution of Bach2 according to the criteria as shown in A; C > N, white bar; C = N, gray bar; C < N, black bar. Cells were counted three times in the different microscopic fields. Averages in the triplicate assays are shown.

nuclear matrix (*14*). To investigate the subnuclear localization of the Bach2 focus, we fractionated the lysate of the HEK293T cells transfected with Bach2, HA-SMRT$^{1205–1668}$ and HDAC4-FLAG, and determined the amounts of proteins in each fraction (Fig. 5). A significant portion of Bach2, HDAC4 and SMRT proteins were detected in the nuclear matrix fraction, in which lamin B, a nuclear matrix marker, is enriched. The endogenous MafK protein, a heterodimeric partner of Bach2, was also detected in the same nuclear matrix fraction. These results suggest that the Bach2 foci are tightly associated with the nuclear matrix *in vivo*.

Effect on de novo mRNA Synthesis—To investigate the biological consequence of the Bach2 focus formation, we performed scratch transcriptional labelling experiments using GM02063 cells as described previously (*8*). The BrUTP incorporation into mRNA during 5 min prior to fixation was visualized with immunofluorescence, and serial confocal images were collected. Although the uptake of BrUTP into mRNA was observed widely in the nucleus, a marked decrease in the incorporation of BrUTP was detected both in and around the Bach2 foci (Fig. 6). The 3D-reconstruction image from the confocal sections clearly showed that transcription activity in the region surrounding the nuclear foci was markedly suppressed. A similar tendency was also observed in cells expressing HDAC5, another class IIa HDAC member (data not shown).

Effect on MARE Sequence-driven Transcription—The above observation raised a possibility that SMRT and HDAC4 promote the Bach2-mediated transcriptional repression by enhancing the focus formation. To test this possibility, we established a model system to detect the Bach2-mediated transcriptional repression by stably transfecting the MARE-driven luciferase reporter gene into the 293T cell chromosomes. Thus obtained cell lines containing the chromosomally integrated reporter were named RBGP2. The RBGP2 cells were transiently transfected with the expression plasmids with several combinations, and then the luciferase activity was measured with a luminometer. Probably because of the presence of AP-1 binding site within MARE sequence, a high basal promoter activity was detected in the RBGP2 cells. In these cells, the Bach2 expression reduced the activity to ~70% of control. However, the simultaneous expression of both HDAC4 and SMRT$^{1205–1668}$ further repressed the MARE-driven luciferase activity in the Bach2 expressing cells, although each one of these two proteins alone failed to cause the synergistic repression (Fig. 7B). This tendency to repress the MARE-regulated transcription was commonly observed among all the three independent cell clones tested (data not shown). Furthermore, we tested whether the MARE is associated with the focus by ChIP experiments. The RBGP2 cells were transiently transfected with the three expression plasmids of Bach2, HDAC4-FLAG and HA-SMRT$^{1205–1668}$, and then the cell lysate was immunoprecipitated with an anti-FLAG, an anti-HA or an anti-MafK rabbit polyclonal antibody. The anti-MafK antibody efficiently concentrated the DNA fragment with the MARE sequence. Although HDAC4 cannot bind directly to any DNA sequences, the MARE sequence was significantly enriched in the immune complex with the anti-FLAG antibody in comparison with the control serum. The interaction between HA-SMRT and the MARE sequence was also detected by using the anti-HA antibody (Fig. 7C).

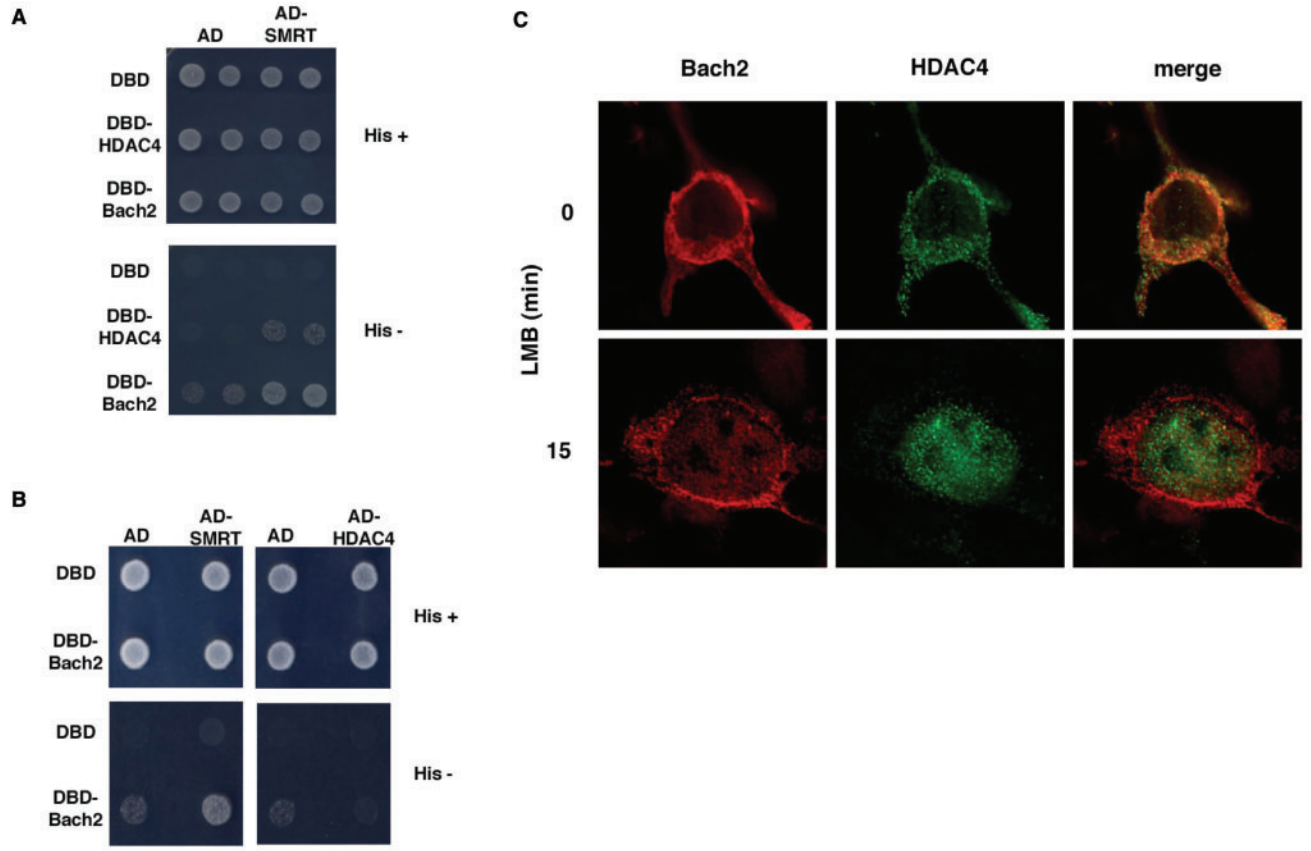

Fig. 4. **Bach2 associates with HDAC4 via SMRT.** (A) Yeast two-hybrid analysis performed with several combinations of plasmids for the GAL4 DNA-binding domain (DBD) and the activation domain (AD) fusion proteins. The resulting transformants were examined for the His^+ phenotype by spotting onto His^- and His^+ plates. DBD-HDAC4 or DBD Bach2 was used as a bait for detecting interaction with AD-SMRT. (B) Yeast two-hybrid for reciprocal analysis. DBD-Bach2 was used as a bait for the interaction with AD-SMRT or AD-HDAC4. (C) Comparison of the nuclear import rate between Bach2 and HDAC4. HEK293T cells co-transfected with Bach2 and HDAC4-FLAG were treated with 10 ng/ml LMB for 0 or 15 min. The proteins were visualized by immunostaining using anti-Bach2 and anti-FLAG (M2) antibodies.

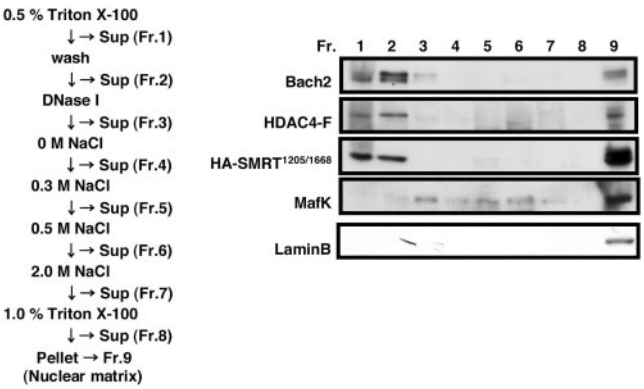

Fig. 5. **Subnuclear localization of Bach2 focus components.** HEK293T cells were co-transfected with Bach2, HDAC4-FLAG and HA- $SMRT^{1205–1668}$, and then the cell extract was fractionated as described in Materials and Methods section. Proteins from each fraction were analysed by immunoblotting using anti-Bach2, anti-FLAG, anti-HA, anti-MafK and anti-Lamin B antibodies. The lane numbers correspond to the fraction numbers. Lamin B is a specific marker for the nuclear matrix.

These observations support the idea that the chromosomally integrated MARE is enriched in the Bach2/SMRT/HDAC4 complexes in the nucleus.

DISCUSSION

The nucleoplasm contains various classes of nuclear bodies, including nucleoli (*1*), Cajal bodies (*3*), nuclear speckles (*2*) and PML bodies (*38*). These higher-order nuclear architectures are supposed to be related with nuclear functions (*39*, *40*). For example, the PML body may serve as a transcriptional activation-related nuclear domain, which assembles transcription activators and co-activators. On the other hand, it has been shown that HDACs and transcriptional co-repressors form transcriptional repression complexes, and are distributed to the nuclear domains named MAD body (*13*, *14*). It has been postulated that the accumulation of nuclear factors in distinct nuclear bodies helps generate a high local concentration of components and hence enhances the efficiency of reactions. Therefore, even in the absence of membraneous compartments, specific mechanisms for compartmentalization must operate within the nucleus

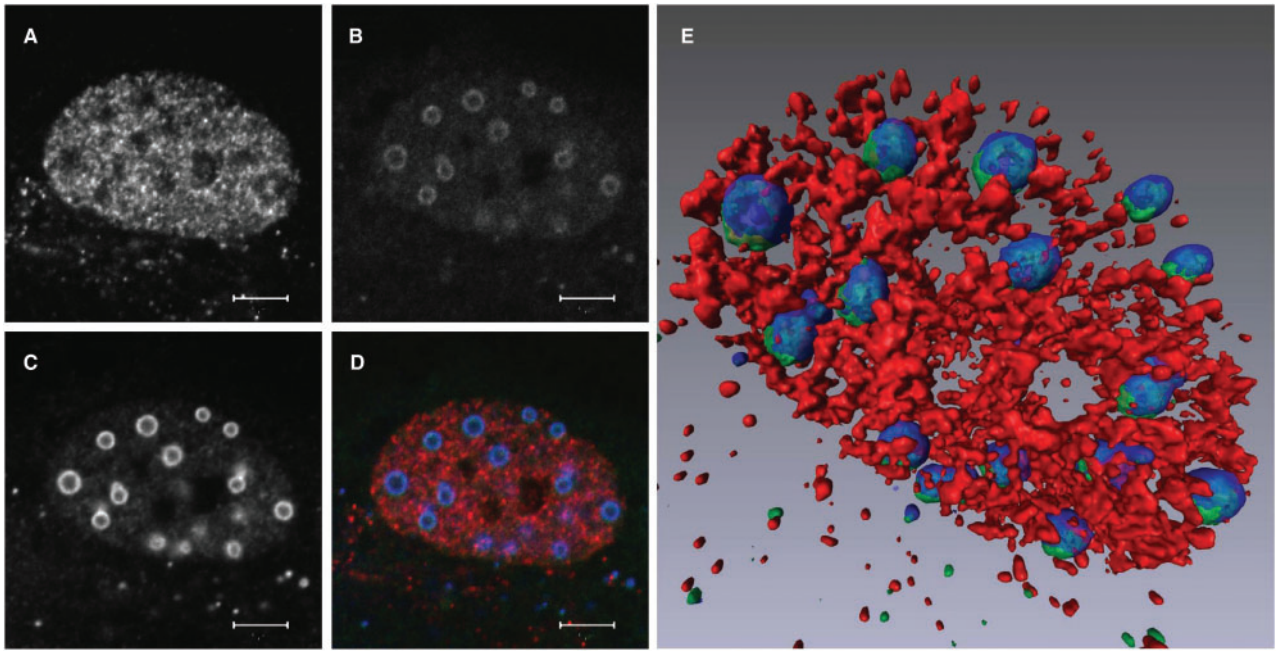

Fig. 6. **Effect of Bach2 focus formation on local transcription.** GM02063 cells were transiently transfected with expression plasmids for Bach2, HDAC4-FLAG and HA-SMRT$^{1205–1668}$. Scratch transcription labelling for 5 min with 1 mM BrUTP was carried out with the transfected cells. Transcription sites, Bach2 and HDAC4 are represented in red (A), green (B) and blue (C), respectively. (D) Bach2 (green) appears to co-localize with HDAC4 (blue). (E) Co-localization of Bach2 and HDAC4 was confirmed in the 3D image. The 3D surface-rendered image was generated with Amira software (TGS) by reconstructing the optical sections. The difference of the surface of Bach2 (green) and HDAC4 (blue) signals is due to the threshold setting for green and blue signals used in the 3D reconstruction.

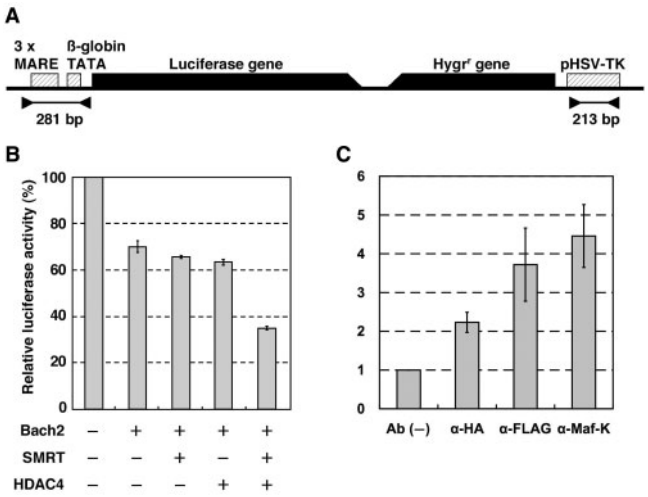

Fig. 7. **Effect of Bach2 focus formation on MARE regulated transcription.** (A) A schematic diagram of the reporter plasmid introduced into the RBGP2 cell lines. The TK promoter has no MARE sequence. Black triangles and lines represent PCR primers for the ChIP experiment and the putative PCR products, respectively. (B) Relative transcription activity. The RBGP2 cells were transiently transfected with the expression plasmids with several combinations, and then the luciferase activity was measured. (C) ChIP analysis. The RBGP2 cells were co-transfected with expression plasmids for Bach2, HA-SMRT$^{1205–1668}$ and HDAC4-FLAG. ChIP experiments were performed with a rabbit serum [Ab (–)], an anti-FLAG, an anti-HA or an anti-MafK antibody. Note that both SMRT and HDAC4 are unable to bind directly to any DNA sequences.

by controlling the assembly of nuclear bodies and targeting factors to the proper locations.

Recently, it was shown that Bach2 foci surround PML bodies and repress the PML body-specific transcription under oxidative stress (*8*). This is probably the first example that one of the nuclear architectures directly affects the function of another class of nuclear domains. It supports the assumption that these nuclear domains are dynamic higher-order nuclear structure rather than static one (*8*). However, we had only limited information about what components consist of the Bach2 focus and how the focus is formed. The present study showed that the co-repressor SMRT and HDAC4 are coordinately involved in the Bach2 focus formation. Our co-expression study suggests that SMRT is essential for the Bach2 focus formation, while HDAC4 alone failed to form the focus. On the other hand, in the presence of SMRT, HDAC4 enhanced the nuclear localization and the focus formation of Bach2. Experiments using LMB, a specific inhibitor of Crm1-mediated nuclear export, demonstrated that the nuclear import of Bach2 was not enhanced by HDAC4, suggesting that HDAC4 increases the retention of Bach2 at the foci. Bach2 did not bind directly to HDAC4, whereas SMRT could bind both Bach2 and HDAC4. These results suggest that the nuclear domain containing SMRT has a potential to form Bach2 focus, but the association with class II HDACs like HDAC4 increases the affinity to Bach2 (Fig. 8).

The BTB-type transcriptional repressors like PLZF and Bcl6 interact with SMRT in a BTB domain-dependent manner. The BTB domain in Bach2 is also important for

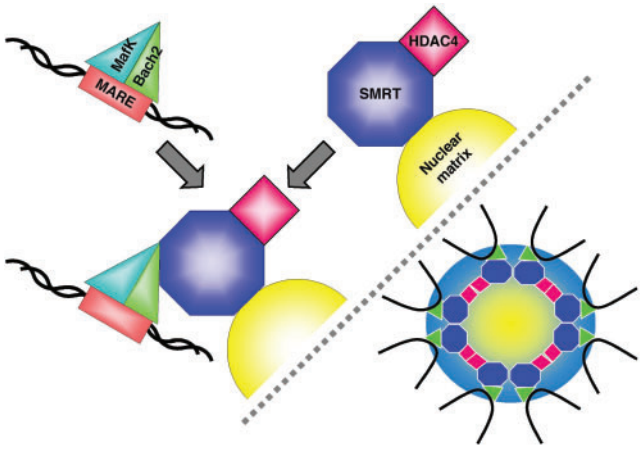

Fig. 8. **The Bach2 focus as a transcription repression domain in the nucleus.** A model for the focus formation. SMRT may be a core component of the Bach2 focus that associates with the nuclear matrix. The Bach2/SMRT/class II HDACs complexes may recruit the MARE-containing DNA regions to the transcription repression domain in the nucleus.

the SMRT binding and the Bach2 focus formation (*8*). The nuclear subdomain containing SMRT/N-CoR and HDACs are known as the MAD body, which is associated with the nuclear matrix (*13*). Indeed, we also showed that the Bach2 focus components are present in the nuclear matrix fraction, indicating that the Bach2 focus is related to the MAD body. The MAD body may function as a scaffold for accumulation of transcriptional repressors. This idea was supported by the present results. We demonstrated that the transcription around the foci was suppressed (Fig. 6) and that there was a decrease in the transcription activity of the chromosomally integrated reporter gene (Fig. 7). These results strongly suggest that the Bach2 nuclear body containing SMRT and class II HDACs functions as a core of the transcription repression domain in the nucleus (Fig. 8).

Recently, it was reported that Bach2 gene deletion occurs in Raji cells, which were established from a Burkitt lymphoma tissue (*41*). In contrast, STI571, an inhibitor of Bcr-Abl kinase, specifically induces the Bach2 expression in chronic myeloid leukaemia (CML) cells (*42*). Moreover, it is also shown that Bach2 promotes oxidative stress-induced cell death (*9*, *43*). These observations suggest the possibility that Bach2 functions as a positive regulator of cell death and may act as a tumour suppressor. It seems possible that the Bach2 focus has a role in the possible tumour suppressor function and regulates oxidative stress-induced cell death. In contrast, other members of BTB-type transcriptional repressors like PLZF and Bcl6 may have an oncogenic activity. Thus, the biological function of the Bach2 focus and distinct roles for the nuclear bodies should be further examined in the near future.

We thank Dr E Seto and Dr E Verdin for supplying the HDAC cDNAs. This work was supported in part by the CREST Research Project, Japan Science and Technology Corporation, a special grant for Advanced Research on Cancer, and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture Sports, Science and Technology, of the Japanese Government.

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