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REGULATION OF HUMAN RNA POLYMERASE III TRANSCRIPTION BY RB FAMILY MEMBERS

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REGULATION OF HUMAN RNA POLYMERASE III TRANSCRIPTION BY RB FAMILY MEMBERS

By

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ABSTRACT

REGULATION OF HUMAN RNA POLYMERASE III TRANSCRIPTION BY RB FAMILY MEMBERS

By

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In humans, the Retinoblastoma tumor suppressor (RB) and its homologues, p107 and p130, can repress transcription by RNA polymerases (Pol) I, II and III of genes whose products are closely linked to cell cycle progression, cell differentiation, cell apoptosis and cell growth.

For Pol III repression by RB, one mechanism elucidated for tRNA gene regulation is that RB targets the transcription factor TFIIIB to interrupt Pol III recruitment to the tRNA promoter. However, RB represses U6 snRNA Pol III transcription by a distinct mechanism, wherein RB, snRNA activating protein complex (SNAPc), TFIIIB, and Pol III co-occupy the U6 snRNA promoter during repression.

Herein I demonstrate that p107 and p130 also are able to repress U6 snRNA transcription *in vitro*. All RB family members directly interact with multiple subunits of the U6 snRNA general transcription machinery including SNAPc and TFIIIB. Furthermore, endogenous RB family proteins associate with Pol III and the A domain of RB is sufficient for RB binding to Pol III. Combined with the phenomenon that RB and Pol III co-occupy the repressed U6 snRNA promoter, a tether and immobilization model is proposed to explain how RB family members repress U6 snRNA transcription.

To my dear family

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LIST OF ABBREVIATIONS

Ad ML	Adenovirus Major late
Bdp1	B double prime
Brf	TFIIB related protein
Brg1	Brahma-related gene 1
Chip	Chromatin immunoprecipitation
DSE	Distal sequence element
EtBr	Ethidium Bromide
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HMTS	Histone methyltransferases
ICR	Internal control region
PIC	Preinitiation complex
Pol	RNA polymerase
PSE	proximal sequence element
PTF	Proximal element transcription factor
RB	Retinoblastoma tumor suppressor protein
RPB1	RNA polymerase II subunit 1
RPC1	RNA polymerase III subunit 1
rRNA	Ribosomal RNA

S-190	Chromatin assembly S-190 extract
SNAPc	Small nuclear RNA activating protein complex
snRNA	Small nuclear RNA
TBP	TATA binding protein
TFIIIA	Transcription factor III A
TFIIIB	Transcription factor III B
TFIIIC	Transcription factor III C
TSA	Trichostatin A

CHAPTER 1

LITERATURE REVIEW

Retinoblastoma (RB) protein, a tumor suppressor

The concept of tumor suppression has been developing for several decades since first suggested in cell fusion experiments (43), wherein the tumorigenetic character of cancer cells was suppressed to some degree after fusion with normal cells. Human retinoblastoma, a rare pediatric eye tumor, has been extensively researched in correlation with the concept of "tumor suppression". After a statistical analysis of retinoblastoma patients, a "two-hit" hypothesis was proposed to explain the formation of retinoblastoma, in which two mutations are required for disease initiation (66). This "two-hit" model was further explained as inactivation of both alleles of a single gene that was responsible for the suppression of retinoblastoma (19). Soon after, a retinoblastoma susceptibility gene, which was abnormal or deleted in some cases of retinoblastoma patients was mapped to chromosome 13q14 (4, 32, 114). Compelling evidence for the existence of a retinoblastoma tumor suppressor was provided when the retinoblastoma (RB) gene was finally cloned and found mutated or deleted in all retinoblastoma tumor samples but not in samples from normal tissues (33, 35, 76). Subsequently, RB was found to play a role in other cancers besides retinoblastoma, includes osteosarcomas, soft tissue sarcomas, leukemias, and many other tumors, where RB is also mutated or inactivated (8). Additionally during infection by different DNA tumor viruses, RB is inactivated by viral oncoproteins, including the Simian virus 40 large T antigen, adenovirus E1A protein, and human papilloma virus E7 protein (21, 25, 127). Together, these observations indicated that RB is a tumor suppressor that in its wild-type form prevents neoplasm.

Abnormal proliferation is a typical characteristic of cancer. In agreement with its role as a tumor suppressor, RB was identified as crucial for cell cycle progression control (123). Overexpression of RB results in the cellular arrest at G1 phase of the cell cycle (40). RB controls the G1/S transition by repressing the transcription of genes whose products are required for S phase entry, including cyclinE, Myc, cdc2, PCNA and others (9, 123). During early G1 phase, RB is hypophosphorylated whereas during late G1 phase, RB is increasingly hyperphosphorylated by cyclin D/cdk4, cyclin E/cdk2, and cyclin A/cdk2 complexs (1, 50). Consequently during early G1 phase, hypophosphorylated RB binds to E2Fs and represses E2F dependent transcription, while during the late G1 and S phase, E2Fs are released from hyperphosphorylated RB and can stimulate target gene transcription (1, 80, 124).

In addition to its function as a tumor suppressor, RB also plays significant roles in tissue development, which was first realized in RB knock out mice (12, 59, 60, 73). While $RB^{+/-}$ mice are predisposed to pituitary gland tumors (12, 60, 73), which confirmed RB as a tumor suppressor, $RB^{-/-}$ knock out mice were non-viable and died between days 13 and 15 of gestation with obvious defects in the development of erythroid, nervous system, lens, and skeletal muscle (12, 59, 60, 73, 134). In $RB^{-/-}$ mice these tissues were not able to withdraw from the cell cycle during terminal differentiation and underwent extensive apoptosis (82, 90, 134). RB was shown to serve as co-activator of some tissue specific transcription factors that are required for terminal differentiation, such as MyoD in the muscle development, and CBFA1 in osteogenic differentiation (41, 117).

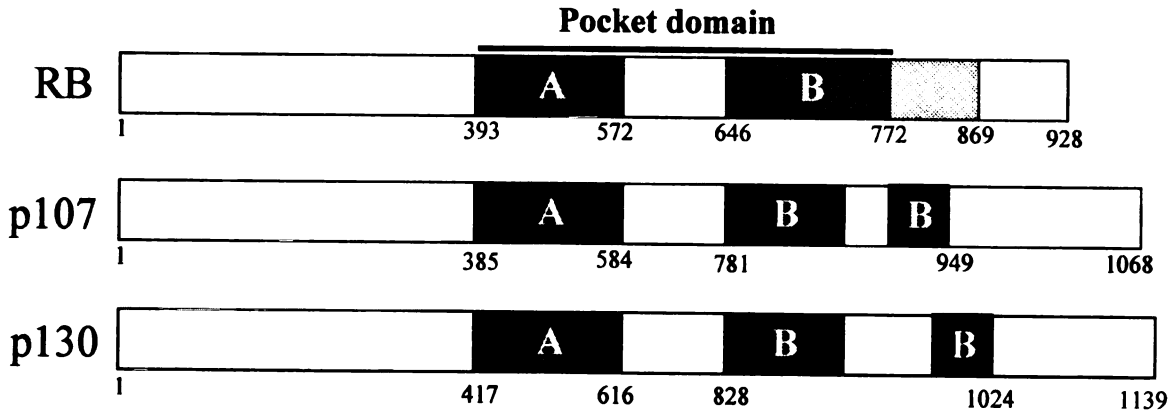
The pocket domain family

RB, p107 and p130 comprise the "pocket" domain family of proteins. These proteins are conserved in both sequence and structure (Fig. 1-1). The "pocket'' family proteins are composed of a divergent N-terminal region, a conserved pocket region, and a C-terminal region. The pocket region consists of two conserved domains, A and B, which are separated by a variable spacer region (26, 84, 98). The pocket region is more highly conserved among pocket family members than the N- or C-terminal regions. There is about 50% amino acid identity between p107 and p130 pocket regions, which is higher than the 30-35% identity of p107 and p130 with RB (78, 129, 138). Both p107 and p130 contain a conserved sequence in the spacer region that is not found in RB, and this sequence is required for strong binding by cyclin A/CDK2 and cyclin E/CDK2 complexes (42, 77). The pocket domain is critical for most functions of RB family members and contains multiple binding sites for target proteins. E2F factors bind to the cleft formed by A and B domain of RB, whereas the LXCXE motif in other proteins, such as the adenovirus E1A and HPV E7 antigen binds to the B domain. The A domain is also required for high affinity binding of these proteins to the B domain (74, 131), suggesting the integrity of pocket domain is important for most RB functions.

RB family members have both common and specialized functions during cell growth. All RB family members can be inactivated by viral oncoproteins, such as the adenovirus E1A, SV40 large T antigen and HPV E7 antigen (92). RB, p107 and p130 all serve as negative regulators of cell proliferation (92), as independent overexpression of each member in the Saos-2 human osteosarcoma cell line consistently inhibits cell

Fig. 1-1. Schematic drawings of RB, p107 and p130. The amino acid positions of the pocket domains are indicated.

Fig. 1-1


Pocket domain
RB
A
B
1
393
572
646
772
869
928
p107
A
B
B
1
385
584
781
949
1068
p130
A
B
B
1
417
616
828
1024
1139

proliferation and causes G1 phase arrest (15, 138). However, inhibition of cell proliferation is cell line specific. Proliferation of C33A human cervical carcinoma cells is inhibited by over expression of p107 and p130, but not by RB (138). In contrast, growth of T98G human glioblastoma cells is inhibited by p130, but not by RB and p107 (69). Whereas a high frequency of mutations in the RB gene has been observed in tumors, p130 gene mutations was rarely found. Until now, mutations in the p130 gene have been detected only in lymphomas, lung cancer and nasopharyngeal carcinoma (11, 16, 99). To date, p107 gene mutations in tumors have not been reported (99).

RB, p107 and p130 all can bind E2F activator proteins and repress E2F-dependent transcription (99). However, each member of the RB family selectively interacts with distinct members of the E2F family. RB binds to E2F-1, E2F-2, E2F-3 and E2F-4, whereas p107 and p130 exclusively bind to E2F-4 and E2F-5 (13). E2F-6 and E2F-7 have not been observed to interact with any RB family members (2). E2F-4 and E2F-5, but not E2F-(1-3), are rich in quiescent cells and the p130/E2F-4 complex accumulates in differentiated cells, suggesting that p130 is a co-repressor of E2F-4/-5 in G0 cells (58, 113). Furthermore, the expression pattern of RB family members during the cell cycle is different. RB expression levels are steady during each phase of the cell cycle (22). In contrast, p130 exhibits maximal expression during the G0 phase of quiescent or differentiated cells (72, 113), whereas p107 expression during the G0 phase is hardly detectable, but its expression levels dramatically increase when cells are stimulated to enter S-phase (6). This expression pattern correlates somewhat with their binding activities to E2F family members. RB binds to E2Fs in both quiescent cells and G1 phase

of cycling cells, p130 forms complexes with E2F-4/-5 in quiescent cells, and p107 binds to E2Fs mainly in the S-phase of cycling cells (137).

The redundant and specialized functions of RB family members were also displayed in mice whose RB family members were knocked out either alone or in combination. As mentioned, $RB^{-/-}$ knock out mice were non-viable coupled with the pronounced defects in the erythroid, nervous system, lens and skeletal muscle development (12, 59, 60, 73, 134), whereas deleterious effects brought by p107 and p130 knock-out are strain dependent. Mice with C57BL/6J genetic backgrounds having either $p107^{-/-}$ or $p130^{-/-}$ knock out are viable, fertile, and show no overt phenotype (17, 75). However, in the BALB/cJ genetic background, $p107^{-/-}$ mice display impaired growth, diathetic myeloproliferative disorder, and short G1 phase (70). $p130^{-/-}$ mice show growth arrest and early embryonic death (71). $RB^{-/-}$ /$p107^{-/-}$ and $RB^{-/-}$/$p130^{-/-}$ double knock out mice have similar phenotypes as the $RB^{-/-}$ mice with similar genetic backgrounds, but die 2 days earlier during embryogenesis (79). Compared with $RB^{+/-}$ mice, both $RB^{+/-}$/$p107^{-/-}$ and $RB^{+/-}$/$p130^{-/-}$ chimera mice have a broader spectrum of tumors, indicating that RB family members may cooperate to suppress tumor formation in different tissues (20).

The molecular mechanisms of transcriptional repression by RB, p107, and p130

Among the 100 or more identified RB binding partners, the majority are transcription factors, including components of the basal transcriptional machinery, chromatin modifiers, and chromatin remodeling factors (91). The function of these factors for RB activity remains, in most case, unproven, In contrast, the mechanism of repression by RB

family members has been mostly characterized in the E2F dependent RNA polymerase (Pol) II transcription.

Multiple mechanisms for E2F repression by RB have been described. First, RB binds to the E2F activation domain and directly blocks its transcriptional activity (30, 44, 49). Second, RB is recruited by E2F family members and then prevents the formation of preinitiation complex (PIC) at the core promoter region (105). Third, RB can actively modify the chromatin structure of the promoter region to repress transcription (14). Nonetheless, *in vivo*, the mechanism for repression of E2Fs dependent transcription remains unclear, and may depend on different promoter contexts. For example, if E2F is the only transcription activator for a specific gene, it may be enough to negatively block the E2F activation domain, whereas, if there are some other transcription factors available besides E2F, blocking E2F may not be enough to repress transcription. Thus, some active repression mechanism by RB may be required. Each of these mechanisms is discussed in more detail in the below.

i. RB repression via direct inhibition of the E2F activation domain. The sites within E2F-1 for RB binding were mapped to the acidic activation region of E2F-1 (30). It was further elucidated by crystal structure analysis that the E2F-1 acidic region binds to the cleft formed by A and B domains of RB (131). The RB-E2F complex remains able to bind to E2F binding sites with target promoter (49), Additional evidence suggests that the interaction between E2F and RB is crucial for RB to repress E2Fs dependent transcription. The interaction between RB and E2F-1 coincides with the repression of

E2F-1 dependent genes (49). Point mutations in E2F-1 that abrogate binding with RB, also make E2F-1 dependent transcription unresponsive to RB repression (30).

ii. RB repression via prevention of preinitiation complex (PIC) formation. When RB is tethered to GAL4, Sp1 promoted transcription of reporter genes containing upstream UAS sequences is repressed (105), which suggests RB can also actively repress transcription via other mechanism than the E2F activation domain occlusion. Thus, the consequence of RB interference with E2F may be the disruption of pre-initiation complex (PIC) formation. In a minimal *in vitro* reconstituted transcription system, RB recruited by E2F prevents the formation of TFIID-A complex, which is an important step for Pol II recruitment (105). However, if RB was added after the formation of TFIID-A complex, E2F dependent transcription becomes resistant to repression (105), suggesting prevention of pre-initiation complex (PIC) formation is an important step to repress transcription.

iii. RB repression via modification of chromatin structure. The effects on transcription brought by posttranslational histone modification and chromatin remodeling have been widely determined (61, 94). The combination of histone H4 K8 acetylation, H3 K14 acetylation, and H3 S10 phosphorylation is often correlated with transcription (61, 101). Acetylation and phophorylation is presumed to neutralize the positive charge of the lysines on histone tails and then loosen the tight binding between the histones and DNA. Loosening DNA from chromatin will ease the recruitment of transcription factors and RNA polymerase (61, 101). Conversely, the lack of H3 and H4 acetylation and tri-methylation of H3 K9 is connected to transcriptional repression (61, 101). Levels

modified histones are controlled by a set of histone modifiers that are functionally antagonistic to each other, such as histone acetyltransferases (HATs) and histone deacetylases (HDACs), and methyltransferases (HMTs) and potential histone demethylases (61, 101, 111). Selective recruitment of those histone modifiers can change local histone modification status.

RB was found to interact with HDAC1-3 of the 7 known HDACs (81, 91). p107 and p130 are also able to recruit HDACs to repress E2F dependent transcription (28). HDAC1 and HDAC 2 have a conserved RB binding motif, LXCXE (91), which binds to a different site on RB than E2F does. Thus, RB may construct an E2F-RB-HDAC repressing complex on E2F dependent genes. RB recruited HDACs are able to reduce the acetylation level of histones at the promoter region on some cell cycle related genes, such as cyclin E, topoisomerase IIα, and thymidylate synthase. The HDACs activity inhibitor, trichostatin A (TSA) reverses histone acetylation levels and compromises RB repression on some genes (112). RB interacts with HDACs in a cell cycle dependent manner. Phosphorylation of RB by cyclin D/cdk4 abrogates HDACs binding (29, 135). However, not all genes, such as Cyclin A, are repressed by RB recruited HDACs, (112, 135). Those genes not influenced by HDACs, may require SWI/SNF or other factors to help repression (see below). It seems that RB family member mediated deacetylation is a fundamental step during transcription of some target genes.

Another behavior coupled to RB induced repression is the induced methylation of nucleosomes on promoters containing E2F binding sites (96, 120). Imunoprecipitated RB

is able to catalyze histone H3-K9 methylation via a RB associated histone methyl-transferase (HMT) called SUV39H1 (96). Overexpression of both RB and SUV39H1 enhances cyclin E repression, whereas SUV39H1/SUV39H2 double knock out mice show increased cyclin E expression (96). Analysis of the H3-K9 methylation levels by Chromatin immunoprecipitation (Chip) assay revealed detectable methylation of histones at the cyclin E promoter in RB $^{+/+}$ cells but not in RB$^{-/-}$ cells (96). RB also interacts with Heterochromatin protein (HP1), which specifically binds to methylated histone H3-K9. Correspondingly, HP1 on the Cyclin E promoter is only detectable in RB $^{+/+}$ cells (96). RB may change histone modifications in a stepwise manner, histone deacetylation followed by histone methylation. Actively recruited HMTs by RB may be required for the permanent repression of some genes in differentiated cells (34). p107 and p130 also physically interact with the SUV39H1, suggesting that a similar mechanism may be used for gene repression by these other RB-related factors (95).

Chromatin remodeling is another important component of RB-mediated transcription regulation. ATP-dependent remodeling complexes, such as SWI/SNF complex can change the nucleosome position on DNA templates. The outcome from changes of nucleosome position is either the facilitation or prevention of promoter access by transcription factors and RNA polymerase (94). Yeast having SWI2/SNF2 knock-out, changes gene expression both positively and negatively, suggesting that SWI/SNF is involved in both activation and repression of different target genes (54).
RB, p107 and p130 can associate with histone modifiers and chromatin remodeling factors, whose recruitment contribute to repression (14). RB induced repression can be

contributed by RB recruited Brg1 and Brm, the two largest subunits of human SWI/SNF complexes (24, 118). Both Brg1 and Brm associate with RB, and this association is cell cycle dependent. Brg1 and Brm dissociate from hyperphosporylated RB (24, 118). In the Brg1 and Brm deficient SW13 cell line, accumulated hypophosphorylated RB is not enough to cause cell cycle arrest (135), whereas, co-expression of either Brg1 or Brm with RB enhances repression and cell cycle arrest (135) . Whether Brg1 and Brm directly bind to RB or not have not yet been determined. RB may form either RB-HDAC-hSWI/SNF or RB-hSWI/SNF complexes on E2F binding sites to execute differential repression of cell-cycle regulated genes (135).

Generally, RB can repress E2F dependent repression with different mechanisms, depending on promoter context or cell situation.

Pol III transcription

In eukaryotes, nuclear genes are transcribed by three different but highly related RNA polymeraseses (Pol), I, II and III. Each RNA polymerase selectively transcribes distinct sets of genes (107). Pol I transcribes the large, tandemly repeated, 28S, 18S and 5.8S ribosomal (r) RNA genes, whose products are essential for ribosome structure and enzymatic activity. Pol II transcribes the protein coding genes (mRNA genes) as well as a subset of small nuclear (sn) RNA genes. Pol III transcribes a set of genes whose main common features are that they encode structural or catalytic RNAs such as 5S rRNA, tRNA, U6 snRNA, and 7SK snRNA (37, 107). Herein, most of the discussion will focus on transcription performed by Pol III. So far 16 subunits have been characterized in

active Pol III that supports *in vitro* Pol III transcription (57). Some subunits are the homologues to the subunits of Pol I and II, such as RPC1 (155 KD) and RPC2 (128KD), the two largest subunits of Pol III, while other subunits are commonly shared between the Pol I, II and III, such as RPABC1, 2 and 3 (37, 57). Remaining subunits are dedicated to Pol III (57).

Promoter structures of Pol III transcribed genes.

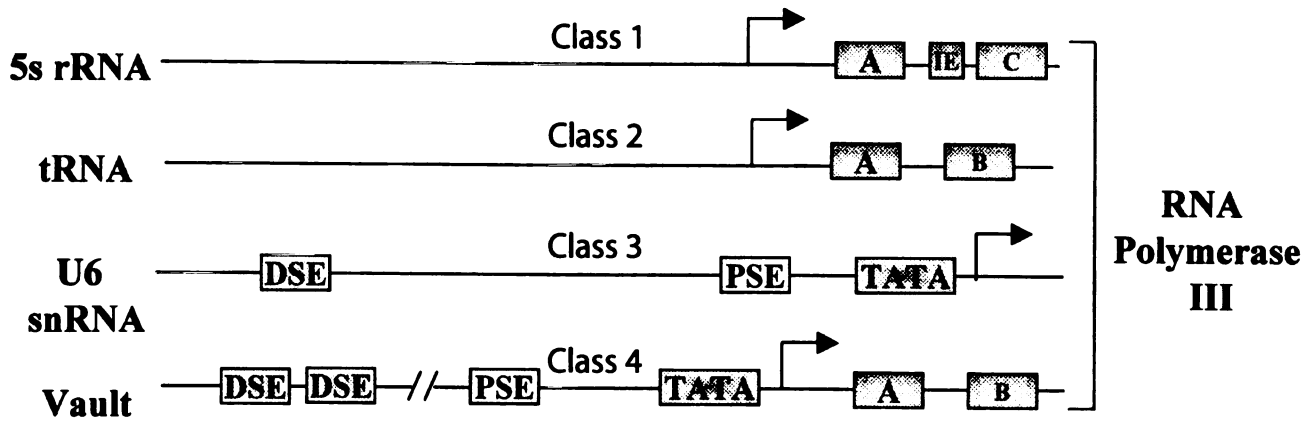
Genes transcribed by Pol III fall into four classes (Fig. 1-2) according to promoter architecture (37, 121).

The promoters of 5S rRNA genes (class1) have a conserved A box, C box and an intermediate elements (IE) (Fig. 1-2) (102, 103). Those elements constitute the internal control region (ICR) that is required for transcription (103). Xenopus laevis 5S rRNA, the best-characterized example, has an A box at +50 to +64, an IE at +67 to +72 and a C box at +80 to +97 DNA sequences (103). Changing the spacing between IE and C box negatively impacts the transcription efficiency (103). Additional upstream DNA sequences may also have some effects on 5S rRNA gene transcription efficiency (104).

The promoters of Ad VA1 and tRNA (class 2) contain a conserved A box and B box down stream of the transcriptional start site (Fig. 1-2) (36, 38, 53). Generally, the A box is proximately located at +8 to +19 bp, while the B box is distally located at +52 to +62 bp (38). The DNA sequences of A and B boxes of tRNA genes partly correspond to the tRNA D-loop and T-loop and thus the promoter structures are also intimately linked to

Fig. 1-2. Promoter architectures for different classes of RNA polymerase III transcribed genes. Class 1(5S rRNA) genes have an internal promoter containing the A and C boxes plus the IE element. Class 2 (tRNA) promoters have internal A and B boxes. Class 3 (U6 snRNA) genes have a DSE, PSE, and TATA box for an external promoter. Class 4 (Vault) genes have combined external and internal promoter elements shared by other 3 classes of Pol III transcribed genes.

Fig. 1-2


5s rRNA
Class 1
A
IE
C
tRNA
Class 2
A
B
U6
snRNA
Class 3
DSE
PSE
TATA
Vault
Class 4
DSE
DSE
//
PSE
TATA
A
B
RNA
Polymerase
III

the function of the tRNA, and consequently the A and B boxes are highly conserved in all tRNA genes (38). The spacing between the A box and B box is variable and has no obvious effects on promoter strength. However, in some species, such as yeast and Drosophila, the upstream DNA sequences up to –50 may somewhat influence transcription (38).

Mammalian U6 and 7SK snRNA genes have typical class 3 promoters (Fig.1-2) (38, 48, 93). In contrast with other Pol III transcribed genes, all the class 3 promoter elements lie upstream of transcribed sequences. The core class 3 promoters contain a TATA box and proximal sequence element (PSE), which are centered around –27 bp and –56 bp respectively (38). Besides the core promoter region, an enhancer site named the distal sequence element (DSE) is present around –239 bp (48). The DSE can be recognized by several transcription factors including Oct-1and Staf (48). So far as known, the DSE and PSE sites are present at all other U-rich snRNA genes (48). Those U-rich snRNA genes that do not have TATA box are transcribed by Pol II (48).

Class 4 genes have combined external and internal promoter whose elements are found in the other three classes (Fig. 1-2). A typical class 4 promoter structure exemplified by Vault genes has an external PSE, DSE, and TATA box along with internal A and B boxes (119, 121). As another example, the Epstein-Barr virus small RNA (EBER) gene has both an external TATA box and internal A and B boxes (55).

Basal transcription machinery of RNA polymerase III

Depending upon the promoter architecture for each class of Pol III transcribed genes, specific basal transcription machinery is required (Fig. 1-3) (48, 100). Class 1 genes require TFIIIA, TFIIIB and TFIIIC (100), whereas class 2 genes require only the TFIIIB and TFIIIC complexes (100). The basal transcription machinery requirements for class 3 genes are different. Class 3 genes require a variant TFIIIB complex and the snRNA activating proteins (SNAPc), which is commonly shared by other Pol II transcribed snRNA genes (48). So far, the components of basal transcription machinery for class 4 genes are not clear but likely components used for other classes are also utilized for these genes. In the following sections, the components of basal transcription machinery will be discussed in more detail.

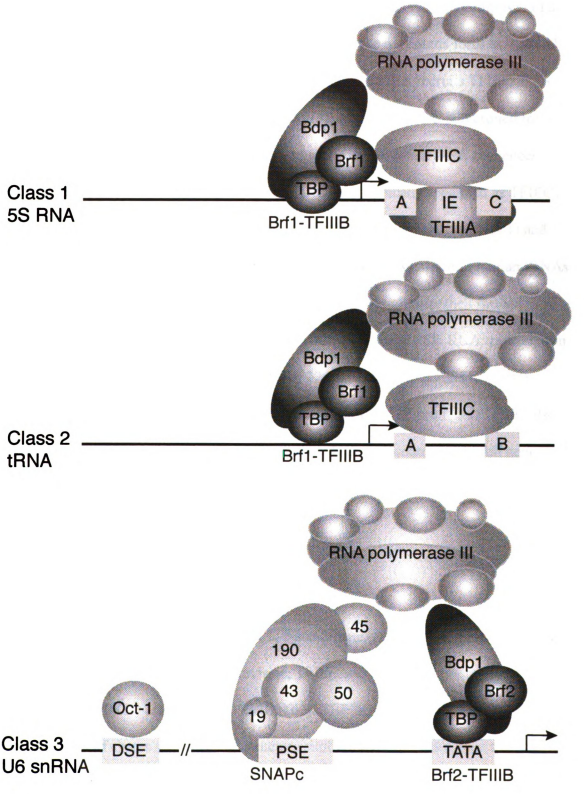
TFIIIA

5S rRNA gene transcription relies on the gene specific transcription factor TFIIIA. Xenopus laevis TFIIIA (39KD) was the first eukaryotic transcription factor to be cloned and was characterized as zinc finger protein that bound DNA specifically (31, 39, 86). Xenopus laevis TFIIIA possesses 9 zinc fingers that span all ICR regions of 5S rRNA genes (31). The first three N-terminal fingers recognize and strongly bind to the C box. The last three C-terminal fingers interact with the A box and the middle three fingers (4-6) loosely contact the intervening promoter region (31). TFIIIA binding on the 5S rRNA gene provides a platform for the productive recruitment of TFIIIC.

TFIIIC

Fig. 1-3. Transcription factors required for transcription by RNA polymerase III. Each identified subunit of transcription factors is indicated. Class 1 (5S rRNA) genes require Brf1-TFIIIB, TFIIIA and TFIIIC. Class 2 (tRNA) genes require Brf1-TFIIIB and TFIIIC. Class 3 (U6 snRNA) genes require SNAPc, Brf2-TFIIIB and Oct-1.

Fig.1-3


RNA polymerase III
Bdp1
Brf1
TBP
TFIIIC
Class 1
5S RNA
A
IE
C
Brf1-TFIIIB
TFIIIA
RNA polymerase III
Bdp1
Brf1
TBP
TFIIIC
Class 2
tRNA
A
B
Brf1-TFIIIB
RNA polymerase III
45
190
Bdp1
Brf2
43
50
Oct-1
19
TBP
Class 3
U6 snRNA
DSE
//
PSE
TATA
SNAPc
Brf2-TFIIIB

TFIIIC is a basal transcription factor that has been widely characterized for its function for 5S rRNA (class 1) and tRNA (class2) transcription (107). The primary function for TFIIIC is to recruit and position TFIIIB (107). In S.cerevisiae, TFIIIC is composed of six subunits, τ138, τ 131, τ 95, τ 91, τ 60 and τ 55 (107). Those 6 subunits form two globular domains and flexible linker region (109). In S.cerevisiae, TFIIIA recruits TFIIIC to 5S rRNA gene promoter through direct interactions (5), whereas, TFIIIC is recruited to tRNA promoters by directly binding to the promoter A and B block DNA sequences (107). In humans, the TFIIIC complex can be chromatographically separated as TFIIIC1 and TFIIIC2 (133). Both TFIIIC1 and TFIIIC2 are required for 5S rRNA (class 1) and tRNA (class 2) transcription (97, 133). The initial promoter recognition of human tRNAs is conducted by TFIIIC2. TFIIIC2 binds to B box of tRNAs promoter then further recruits TFIIIC1 and TFIIIB (97). TFIIIC1 may also help U6 and 7SK RNA transcription (97). TFIIIC2 is comprised of 5 Polypeptides of 220, 110, 102, 90 and 63 kD (107). Besides helping to assemble the pre-initiation complex on Pol III promters, TFIIIC also display intrinsic histone acetyltransferase activity, which may contribute to full gene activity. The TFIIIC110 and TFIIIC90 subunits of TFIIIC were shown to provide histone acetyltransferase activity (56, 68).

TFIIIB

TFIIIB is commonly required for all class 1, 2 and 3 gene transcription. TFIIIB recruits Pol III by direct contact (122). In S.cerevisiae, TFIIIB was identified as a complex of TBP, B' (Brf1, 70KD) and B"(Bdp1, 90KD) (63). TBP is required for most Pol III transcription. Brf shares sequence and structure similarity with TFIIB (18), a factor in the

Pol II basal transcription machinery, and is thus named as TFIIB-related factor (18). In contrast with the single TFIIIB in S.cerevisiae (62), there are different hsTFIIIB variants required for class 1, 2 and 3 gene transcription in humans (107). There are several forms of human Brf, including Brf1, Brf2, and Brf-V2 (a splice form of Brf1), as well as human Bdp1, including Bdp1 and the Bdp1 alternative splice forms, Bdp1-V2 and Bdp1-V3 (128). TFIIIB containing TBP, Brf1 and Bdp1 supports tRNA and 5S rRNA transcription (67, 87). However, class 3 gene transcriptions do not require Brf1 but instead require Brf2 (108). Brf2-v2 may be also required for class 3 genes during in vivo transcription (85). Recombinant TBP, Brf2 and Bdp1 can reconstitute class 3 gene transcription using a minimal in vitro system (57). The roles played by the Bdp1-V2 and Bdp1-V3 splice forms remain to be investigated. TFIIIB is recruited on Pol III promoters through different ways. For class 1 and 2 TATA box less genes, TFIIIB is recruited by direct interaction with TFIIIC (100). For class 3 genes, TFIIIB can directly bind to the TATA box (100). After DNA binding of TFIIIB promotes the DNA melting for formation of the open complex (65).

SNAPc

The transcription of human U6 and 7Sk snRNA genes (class 3) specifically requires the small nuclear RNA activating protein complex (SNAPc). SNAPc is also required for other U-rich snRNA gene transcription by RNA polymerase II. The requirement of SNAPc is due to the external PSE elements on snRNA promoters. SNAPc, also named proximal element transcription factor (PTF), is composed of at least 5 identified subunits: SNAP190 (130), SNAP50/PTFβ (3, 45), SNAP45/PTFδ (106, 132), SNAP43/PTFγ (47,

132) and SNAP19 (46). Immunodepletion of SNAPc from cell extract blocks U1 and U6 snRNA transcription (46). SNAPc specifically binds to PSE DNA sequences; and its binding activity on DNA is increased with cooperation from TBP (88). In an in vitro transcription system, mini-SNAPc comprised of recombinant SNAP190 (1-505), SNAP50 and SNAP43 supports U6 (89) and U1 (L. Gu, personal communication) snRNA transcription. SNAPc cooperates with TFIIIB to recruit Pol III on U6 promoters (88). On a chromatinized template, the direct interaction between Oct-1 on the DSE and SNAP190 on the PSE helps SNAPc to settle on the PSE (136).

The Pol III transcription cycle

Once the preinitiation complex is formed, it takes about 5 minutes at 22 °C for yeast tRNA to complete initiation process, including Pol III recruitment, DNA helix melting and initial RNA synthesis (23). Pol III with the help of TFIIIB melts DNA around start site more than 14bp length, but it is sensitive to temperature (64). After initiation, Pol III starts elongation without an obvious pause and elongates RNA at an average rate of ~20 nt/s at 20 °C in yeast (83). During elongation, the preinitiation complex does not dissociate from the promoter region immediately, and is used to re-initiate several rounds of transcription (23). This reinitiation process, as well as Pol III recycling has been well established in yeast tRNA and U6 snRNA transcription (27). Transcriptional reinitiation strongly enhances the transcription rate by cutting out the relatively long time spent on initiation (23). The termination of Pol III transcription relies on a cluster of T residues (four or more) next to the transcribed region (7).

Regulation of Pol III transcription by RB family members

Abnormal hyperactivity of Pol III was observed in mice with myelomas about three decades ago (110). Those findings prompted investigation into the mechanism for normal Pol III activity in normal cells. Although the connection between the abnormal Pol III activity and cancer is yet to be investigated, the mechanisms for Pol III regulation have advanced significantly since the 1990s (126), and recently RB, p107 and p130 have been closely linked to the regulation of Pol III activity (116, 126). Global Pol III repression by RB, p107 and p130 was observed both in vivo and in vitro (51, 52, 116, 126). Transient overexpression of either p107 or p130 represses Pol III transcription in vivo (116), and in vitro, recombinant p107 and p130 repress Ad VA1, tRNA and 5S rRNA transcription (116). In $RB^{-/-}$ mice primary fibroblasts, Pol III activity, as detected by nuclear run on assay was elevated, whereas general Pol II activity was not affected (126). As with p107 and p130, recombinant RB represses Pol III in vitro transcription (51, 52, 126), whereas a natural occuring mutant RB found in cancer does not repress Pol III transcription (126). Pol III activity varies during cell cycle and has maximal activity at the S and G2 phases with minimal activity at G0, early G1, and M phases (125). This variation in Pol III activity is consistent with the activity of RB. RB loses its repression activity starting during middle G1 phase (123).

For Pol III transcription, different molecular mechanisms for RB repression have been proposed, according to the different features of each class of Pol III transcribed gene. RB was found to interact with both TFIIIB and TFIIIC2 (10), two factors required for class 1 and 2 gene transcription. The interaction between RB and TFIIIB disrupt the contacts

between TFIIIB and other components of basal transcription machinery as well as Pol III, and further prevents the recruitment of Pol III (115). As for U6 snRNA (class 3) gene repression, RB was found to interact with SNAPc, however, RB did not abrogate the Pol III occupation on U6 promoter (51, 52). Those findings suggest RB may repress different class of Pol III genes by different mechanisms.

Summary

RB family members can repress Pol I, Pol II, and Pol III transcription of genes that play essential roles in cell cycle control, cell growth, differentiation and apoptosis. Pol III activity is presumed to regulate cell growth potential, and it is both academically and practically significant to know the molecular mechanism for how RB family members repress Pol III transcription. The following chapter will focus on discussing the repression mechanism of U6 snRNA transcription by RB, p107, and p130.

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CHAPTER 2

REGULATION OF HUMAN U6 snRNA TRANSCRIPTION BY RETINOBLASTOMA FAMILY MEMBERS

Abstract

The Retinoblastoma tumor suppressor (RB) represses U6 snRNA gene transcription by RNA polymerase III (Pol III). Whether the other two RB related family members p107 and p130 regulate U6 snRNA transcription is unknown. My research shows that p107 and p130 repress U6 snRNA transcription at levels comparable to RB. All RB family members directly interact with multiple components of two U6 snRNA transcription complexes, SNAPc and TFIIIB, which are required for U6 snRNA transcription. The U6 snRNA specific TFIIIB is composed of three proteins, TBP, Brf-2 and Bdp1. Interestingly, Bdp1 selectively interacts with RB only. An additional association between RB family members and Pol III was observed and the A domain of RB is sufficient for RB binding to Pol III, which suggests the mechanism for U6 snRNA repression by RB family members could involve direct interactions with the polymerase. Combined with the previous finding that RB and Pol III co-occupy the U6 snRNA promoter during repression, a tether and immobilization model is proposed to explain the mechanism for U6 snRNA transcriptional repression by RB family members.

Introduction

RB family members (RB, p107 and p130) play essential roles in controlling cell growth, proliferation, cell-cycle progression, differentiation and apoptosis (5, 16, 17, 22). Cell cycle control by RB family members is closely connected to their role in regulating the transcription of genes containing E2F binding sites, whose products control the cell cycle transition (1, 31). There are multiple mechanisms adopted by RB to repress E2F dependent Pol II transcription. First, RB blocks E2F transcriptional activity through direct binding to its activation domain (8, 10, 12). Second, RB prevents the formation of the preinitiation complex (PIC) at target gene promoters (21). Third, RB represses Pol II transcription by modifying the chromatin structure (6).

In addition to the regulation of E2F-dependent Pol II transcription, RB family members also regulate the transcription by Pol I and Pol III (3, 4, 13, 32). All RB family members repress Pol III activity both *in vivo* and in *vitro* (2, 13, 35). Pol III activity as detected by nuclear run on assay is elevated in $RB^{-/-}$ mice primary fibrolasts (35). Furthermore, Pol III activity reflected by 5S RNA, tRNA, and Ad VA1 transcription is also down-regulated in cells transiently expressing p107 or p130 (27). Recombinant RB represses *in vitro* transcription of all classes of Pol III genes (13, 19), whereas recombinant p107 and p130 were shown to repress the *in vitro* transcription of Pol III transcribed 5S RNA, tRNA and Ad VA1 gene (27). That p107 and p130 also regulate U6 snRNA transcription has not been demonstrated. The significance of Pol I and Pol III repression by RB is that the Pol I and Pol III products including rRNA, tRNA and U6 snRNA are closely related to protein synthesis and cell growth (33).

One major goal of our laboratory is to understand the mechanism of how RB family members repress Pol III transcription using U6 snRNA transcription as a model. The mechanisms elucidated on Pol II repression by RB are not able to explain Pol III repression by RB. One reason is that Pol III transcription requires different transcription factors than E2F (9).

Based on promoter architecture, genes transcribed by RNA Pol III can be divided into four classes, class 1, 2, 3 and 4 (Fig. 1-2), represented by the 5S RNA, tRNA, U6 snRNA and Vault genes, respectively (9, 11, 30). Except for class 4 genes, the transcription factors required for the other 3 classes genes are well identified (Fig. 1-3). The only member of class 1, the 5S rRNA gene, requires TFIIIA, TFIIIB and TFIIIC. Class 2 genes (tRNA and Ad VA1) require TFIIIB and TFIIIC complexes, whereas class 3 genes (U6 snRNA) require SNAPc and TFIIIB (20). The TFIIIB required by class 1 and 2 genes contains TBP, Bdp1 and Brf1, whereas the TFIIIB required by class 3 genes contains TBP, Bdp1 and Brf2 (23). Consistent with their differing transcription factor requirements, different mechanisms for RB repression of Pol III transcription have been observed. For class 1 and 2 gene repression, RB targets TFIIIB by directly binding to Brf1 or TBP to disrupt the interaction between TFIIIB and TFIIIC (26). For U6 snRNA (class 3) gene repression, RB targets SNAPc via SNAP43 and SNAP50, and TFIIIB via TBP and Bdp1 (13). Interestingly, RB and RNA polymerase III co-occupy a repressed U6 snRNA promoter, suggesting that RB is not necessary to block preinitiation complex

formation at the U6 snRNA gene promoter (14). Instead, the interactions among RB, SNAPc, and TFIIIB may be important for correctly targeting RB to the promoter.

The phenomenon of RB and RNA polymerase III co-occupying a repressing U6 snRNA promoter (14) raises the question, by what mechanism does RB inactivate Pol III on the U6 promoter. Other questions that will be addressed are whether p107 and p130 also repress U6 snRNA transcription and whether all RB family members share common mechanisms and functions for U6 snRNA repression.

RB, p107 and p130 share a conserved pocket A/B domain (Fig. 1-1), which is responsible for most RB family member functions (28). In addition to overlapping functions in cell proliferation control, RB, p107 and p130 maintain some unique features (6). For example, RB binds to E2F1-E2F4, but p107 and p130 bind to E2F4 and E2F5 only (6). The expression pattern of each RB family member during the cell cycle is also different. Protein p130 is highly expressed in quiescent cells, and its expression level decreases rapidly when G0 stage cells are stimulated to enter the cell cycle. In contrast, p107 expression level is very low during the G0 stage of the cell cycle, and its level rises quickly when cells enter into S phase. RB expression levels are moderate and quite stable in each phase of the cell cycle (6). p107 and p130 were also found to repress class 1 (5S RNA) and class 2 (tRNA) gene Pol III transcription by targeting TFIIIB (27). Initial chromatin immunoprecipitation (Chip) assays performed in our lab (unpublished data) showed that p107 and p130 also occupy the U6 snRNA promoter, indicating that p107 and p130 may repress U6 snRNA transcription.

The data presented here show that RB, p107 and p130 all repress U6 snRNA Pol III transcription *in vitro*. RB, p107 and p130 can interact with multiple subunits of SNAPc and TFIIIB including the SNAP190, SNAP50 and SNAP43 subunits of SNAPc, and the Brf1 and TBP subunits of TFIIIB. Interestingly, Bdp1 was found to interact with RB only, the significance of this difference for RB, p107 and p130 repression remains to be investigated. Additionally, endogenous RB, p107 and p130 associate with Pol III and the A domain of RB is responsible for the Pol III association. Combined with the previous finding that SNAPc and TFIIIB bind to RB in a region other than the A domain (14), a tether and immobilization model is proposed to explain how RB inactivates the Pol III on the U6 snRNA promoter during repression.

Materials and Methods

Subcloning of GST-p107 and GST-p130

Partial p107 and p130 sequences were subcloned correspondingly from pCMV-p107 and pDEF3-p130 (36), which contain full length cDNAs encoding p107 and p130. The PCR primers used for p107 subcloning are 5'-GACTAGTCTAGAATTGCTGTACTGTGTGAACTGC-3' and 5'-GACGCGGATCCTTAATGATTTGCTCTTTCACTGAC-3', which correspond to amino acids from 249 to 1068 of p107. The PCR primers used for p130 subcloning are 5'-GACTAGTCTAGAGGTTCAGGAACAGAGACTGCTG-3'and 5'-GACTAGCTAGCCCGTCGGGAGGTGACCAGTCG-3', which correspond to amino acids from 372 to 1139 of p130. The PCR products p107 and p130 were individually

inserted into a pET11c-based expression vectors with an in-frame GST tag fused to the N-terminus of p107 and p130 to make pGST-p107 (249-1068) and pGST-p130 (372-1139). The DNA sequence of pGST-p107 (249-1068) and pGST-p130 (372-1139) was checked by sequencing.

Preparations of recombinant proteins

Recombinant GST-p107 (249-1068), GST-RB (379-928), and GST were expressed in *E.coli* BL21 DE3. GST-p130 (372-1139) was expressed in *E.coli* BL21 $DE3^+$ cultured in the media with 37μg/mL of chloromphenical. Protein expression was induced by IPTG (1mg/mL) for 18 hours at 16 °C. Bacteria were homogenized with Wheaton Dounce Homogenizer (15 mL volume, 25 –30 strokes with B pestle) in HEMGT-150 buffer (25 mM Hepes pH 7.9, 0.5 mM EDTA, 12.5 mM $MgCl_2$, 10% glycerol, 0.1% Tween-20, 150 mM KCl) plus protease inhibitors (1 mM sodium bisulfate, 1 mM benzamidine, 1 μM pepstatin A, 0.5 mM PMSF) and 1 mM DTT. Protein extracts were incubated with glutathione sepharose 4B beads (Amersham Biosciences Inc.) for 4 hours at 4 °C. Beads were washed with HEMGT-150 buffer for 3 times and the bound GST-fusion proteins were eluted with HEMGT-150 buffer containing 50 mM reduced glutathione. The eluted GST fusion proteins were dialyzed against Dignam buffer D (20 mM HEPES, pH 7.9, 0.2 mM EDTA, 5 mM $MgCl_2$, 10% glycerol, 0.1% Tween-20, 100 mM KCl) using a 28-Well Microdialysis System apparatus (GIBCOBRL Inc.). The GST fusion proteins were concentrated to more than 100 ng/μl through YM-30 centricon devices (Millipore Inc. Cat# 4208). The concentration, molecular weight, and purity of GST fusion proteins were checked by SDS-PAGE and Coomassie blue staining.

***In vitro* transcription assay**

To assay repression by RB family proteins, GST-RB family fusion proteins were added to the following *in vitro* transcription systems of U6 snRNA, Adenovirus (Ad) VA1 and Ad major late (ML) as previously described (13, 14). For *in vitro* U6 snRNA transcription, 250 ng of pU6/Hae/Ra.2 and 2 μl HeLa cell nuclear extract were used in a 20 μl reaction. For Ad VA1 *in vitro* transcription, 250 ng of pBSM13+VA1 and 2 μl HeLa cell nuclear extract were used in a 20 μl reaction. For Ad ML *in vitro* transcription, 250 ng M13-Ad ML and 4 μl HeLa cell nuclear extract were used in a 25 μl reaction. All *in vitro* transcription reactions were carried out at 30°C for 1 hour. The transcription levels of Ad VAI and Ad ML were assessed by the amount of incorporated α^{32}P-CTP, and the U6 snRNA transcription levels were assessed by the amount of α^{32}P-CTP radiolabeled RNA probe protected by pU6/Hae/Ra.2 transcripts after being subjected to a T1 RNAase protection assay. Transcripts were separated by denaturing 6% PAGE and visualized by autoradiography.

Co-immunoprecipiation and Western blot assay

Co-immunoprecipitation assays were performed by incubating 600 μl of N2 HeLa cell nuclear extract with 3 μg of goat IgG, anti-p107 (Santa Cruz, sc-318), anti-p130 (Santa Cruz, sc-317), or anti-RB (Santa Cruz, M-15) antibody at 4°C for overnight. Then, for each sample, 1 mL of HEMGT-150 containing protease inhibitors and 1 mM DTT was added together with 25 μl of Protein G agarose beads (Upstate Inc. Cat.# 16-266) for another 4 hour rotation at 4°C. The beads were washed with 1 mL of HEMGT-150 3

times, and the proteins bound to beads were eluted by boiling in Laemmli buffer. The eluted proteins were separated by 7.5% SDS-PAGE followed by transfer to nitrocellulose membrane using a semi-dry transfer method. Proteins on nitrocellulose membrane were detected by Western blot analysis (14) using either anti-Pol III (largest subunit, RPC1) (MI-170), anti-Pol II (8WG16), anti-SNAP43 (CS48), or anti-actin antibodies. In the reciprocal co-immunoprecipitation assay, 600 µl of N2 HeLa cell nuclear extract was incubated with 3 µg of rabbit preimmune serum or rabbit Pol III (largest subunit, RPC1) (MI-170) anti-serum. Co-immunoprecipitated proteins were then detected by Western analysis, using antibodies specific to RB (M-15), TBP (SL2) and actin.

Co-mmuoprecipitation assay with the treatment of Ethidium Bromide

The normal structure of DNA in HeLa cell nuclear extract was disrupted by treating with ethidium bromide (EtBr) (18). EtBr was added to HeLa cell nuclear extract to 100 µg/mL final concentration before being used for co-immunoprecipitation assay. The HeLa cell nuclear extract with EtBr was clarified by centrifugation at 13000 rpm (16,000 g) at 4°C for 5 minutes. 600 µl of clarified HeLa cell nuclear extract was incubated with 3 µg of goat IgG, anti-p107, anti-p130, or anti-RB antibodies at 4°C overnight. Other conditions were same as described above, except 100 µg/mL of EtBr was added to the HEMGT-150 buffer for the wash steps.

GST pull-down assay from HeLa cell nuclear extract

GST fusion proteins used in GST pull-down assays were prepared as described before (14). GST pull-down assays were performed by incubating 300 µl of HeLa cell nuclear

extract with 1 μg of either recombinant GST, GST-RB (379-928), GST-RB containing the A, B and C domains (379-870), GST-RB containing the A and B domains (379-772), GST-RB containing the A domain (379-577), GST-RB containing the B and C domains (645-870), GST-RB containing the B domain (645-772), GST-RB mutant with Δexon 22 (379-928), or GST-RB (379-928) with C706F mutation at 4°C for overnight. Recombinant GST or GST fusion proteins together with associated proteins were precipitated through binding to 25 μl of glutathione sepharose 4B beads (Amersham Biosciences Inc.) by incubation at 4°C for 4 hours. After washing the beads 3 times (1mL per wash) with HEMGT-150 buffer containing protease inhibitors and 1 mM DTT, the bound proteins were eluted by boiling in Laemmli buffer. The eluted proteins were separated by 7.5% SDS-PAGE and then transferred to nitrocellulose membrane. The recovered proteins on nitrocellulose membrane were detected by Western blot using either anti-Pol III (largest subunit, RPC1), anti-Brg1 (H-88), anti-actin or anti-GST antibody. The result of Western blot assay using anti-GST antibody indicated relative amount of GST and GST fusion proteins recovered in the GST pull-down process.

GST pull-down assay with *in vitro* translated proteins

Each subunit of SNAPc, TFIIIB and Oct-1 was individually transcribed and translated in TNT T7 coupled reticulocyte lysate system (Promega Cat. # L4610). During *in vitro* translation, ^{35}S-methione was incorporated into proteins for labeling. Between 3 to 20 μl of crude ^{35}S-labelled protein (depending on the protein concentration) were incubated with 1 μg of GST, GST-p107, GST-p130 or GST-RB in HEMGT-150 buffer at 4°C for 2 hours. Then 1 mL of HEMGT-150 buffer containing protease inhibitors and 1 mM DTT

was added together with 20 μl of glutathione sepharose 4B beads to each sample for another 2 hours. The beads were washed with 1 mL HEMGT-150 buffer for 3 times. Bound proteins were eluted from beads by boiling in Laemmli buffer. Eluted proteins were separated by 12.5% SDS-PAGE. The recovered GST fusion proteins in each sample were monitored through Coomassie blue staining, and the ^{35}S-labelled proteins in SDS-PAGE were detected by autoradiography.

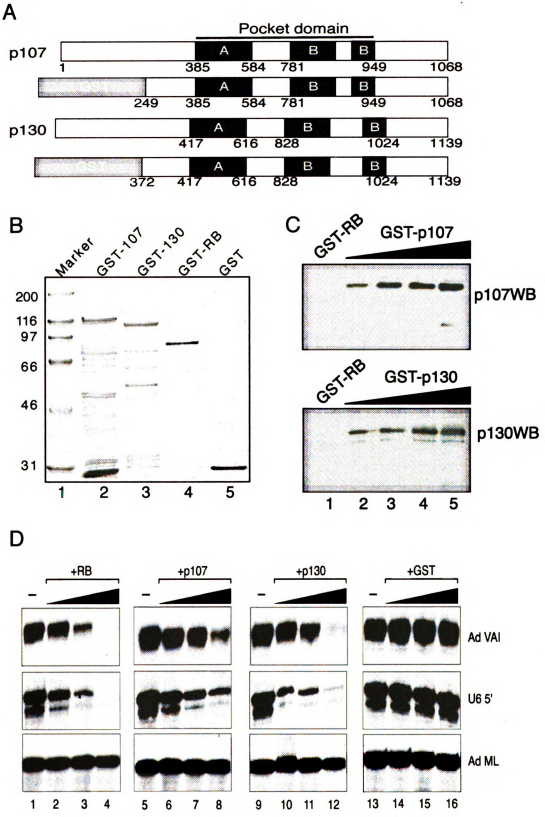
Results

All RB family members repress U6 snRNA transcription *in vitro*

To test whether p107 and p130 are able to repress U6 snRNA (class 3) transcription, *in vitro* repression assays were performed with recombinant GST fusion RB family proteins. The GST-RB (379-928) used here contains the A/B pocket domain and the C-terminal region, which is competent for repression on all three classes of Pol III transcribed genes (14). GST-p107 (249-1068) and GST-p130 (372-1139) used here both contain the A/B pocket domain and C-terminal region (Fig. 2-1 A), which are active for *in vitro* repression on 5S RNA (class 1) and Ad VA1 (class 2) transcription (27). First, the size and purity of GST fusion proteins were checked by SDS-PAGE and Coomassie blue staining. As shown in Fig. 2-1 B, the size of each recombinant protein, compared to protein size standards, matched the expected molecular weight. Smaller molecular weight proteins may be truncated forms of GST-p107 (249-1068) and GST-p130 (372-1139), or other miscellaneous proteins from *E.coli*. The GST-p107 (249-1068) and GST-p130 (372-1139) were not previously expressed in our laboratory, and therefore, the expression of the full-length proteins was further confirmed by Western blot analysis. As shown in

Fig. 2-1. All RB family members are able to repress human U6 snRNA transcription *in vitro*. (A) Schematic representations of GST-p107 and GST-p130. (B) Analysis of recombinant GST-p107 (249-1068) (lane 2), GST-p130 (372-1139) (lane 3), GST-RB (379-928) (lane 4) and GST (lane 5) proteins by SDS-PAGE and Coomassie blue staining. Protein molecular weight marker is shown in lane 1. (C) GST- p107 (249-1068) and GST-p130 (372-1139) are specifically recognized by p107 and p130 antibodies. Western blot analysis was performed using p107 (top gel) and p130 antibodies (bottom gel). Different amounts of recombinant GST-p107 (249-1068) or GST-p130 (372-1139) were loaded (lanes 2-5). GST-RB (379-928) (lanel) was used as a negative control. (D) GST-RB (379-928), GST-p107 (249-1068) and GST-p130 (372-1139) repress the Pol III transcription of Ad VA1 and U6 snRNA genes, but not Pol II transcription of Ad ML gene. Approximately, 0, 200, 400, 800 ng of GST-RB (379-928) (lanes 1 to 4), GST-p107 (249-1068) (lanes 5 to 8), or GST (lanes 13 to 16) were added to *in vitro* transcription assays using HeLa cell nuclear extracts. Lanes 9-12 contain 0, 100, 200, 400 ng of GST-p130 (372-1139). Transcripts were processed as described previously (13, 14).

Fig. 2-1


A
Pocket domain
p107
A
B
B
1
385
584
781
949
1068
249
385
584
781
949
1068
p130
A
B
B
417
616
828
1024
1139
372
417
616
828
1024
1139
B
Marker
GST-107
GST-130
GST-RB
GST
200
116
97
66
46
31
1
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4
5
C
GST-RB
GST-p107
p107WB
GST-RB
GST-p130
p130WB
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Fig. 2-1 C, GST-p107 (249-1068) and GST-p130 (372-1139) were recognized by anti-p107 and anti-p130 antibody, respectively, but GST-RB was not recognized by any one of those antibodies, indicating GST-p107 (249-1068) and GST-p130 (372-1139) were successfully cloned and expressed. Antibodies against p107 and p130 were designed upon the non-conserved C-terminal region, and do not to cross-react with each other.

Next, these proteins were tested for their ability to repress Ad VA1, U6 snRNA, and Ad ML transcription. The *in vitro* repression assay of Ad VA1 transcription served as a positive control for repression activity of the GST fusion proteins, whereas the repression assay on Ad ML transcription served as negative control to indicate that the repression by the GST-fusion proteins was gene specific. As shown in Fig. 2-1 D, both Ad VAI and U6 snRNA gene transcription decreased as increasing amounts of GST-RB (lanes 1 to 4), GST-p107 (lanes 5 to 8) and GST-p130 (lanes 9 to 12) were added in each repression assay. Ad VA1 and U6 snRNA gene transcription were barely detectable when 800 ng of GST-RB (lane 4) or 400 ng of GST-p130 (lane 12) were included in the reactions, although transcription was still detectable when 800 ng of GST-p107 (lane 8) was present in the reaction. As controls, the transcription levels of Ad VA1 (top gel, lanes 13-16) and U6 snRNA (middle gel, lanes 13 to 16) were not affected by addition of an equivalent mass of GST to the reactions. This analysis suggests that GST-p107, GST-p130 and GST-RB can repress Ad VA1 and U6 snRNA gene transcription, and the repression activity is from RB family proteins. As a control of gene specific repression, the Ad ML transcription levels (bottom gel, lanes 1 to 4, 5 to 8, 9 to 12) were not influenced by increasing amounts of GST-RB (lanes 1 to 4), GST-p107 (lanes 5 to 8), GST-p130 (lanes

9 to 12), or GST (lanes 13 to 16), suggesting the repression by GST fusion proteins tested here was also gene specific. The *in vitro* repressions of Ad VA1 by RB family members and the repression of U6 snRNA by RB found here are consistent with previous findings (13, 14). Furthermore, this is the first example of U6 snRNA gene repression by p107 and p130 *in vitro*.

Endogenous RB family members associate with multiple subunits of RNA Polymerase III, SNAPc and TFIIIB, and associations are not DNA dependent

One typical character for RB family protein mediated repression is the stable and direct binding to some RNA polymerase accessory factors, such as E2F (31). This direct and stable binding character for RB family proteins also were found in RB family protein induced Pol III repression (26, 27). Previous findings showed that endogenous RB family proteins associate with TFIIIB (26, 27), and endogenous RB was also found to associate with SNAPc (13). However whether endogenous p107 and p130 associate with SNAPc was not investigated. Another interesting phenomenon is that RB and Pol III co-occupy the repressed U6 snRNA promoter (14), which suggests that Pol III may be retained at the U6 snRNA promoter through some RB mediated mechanism.

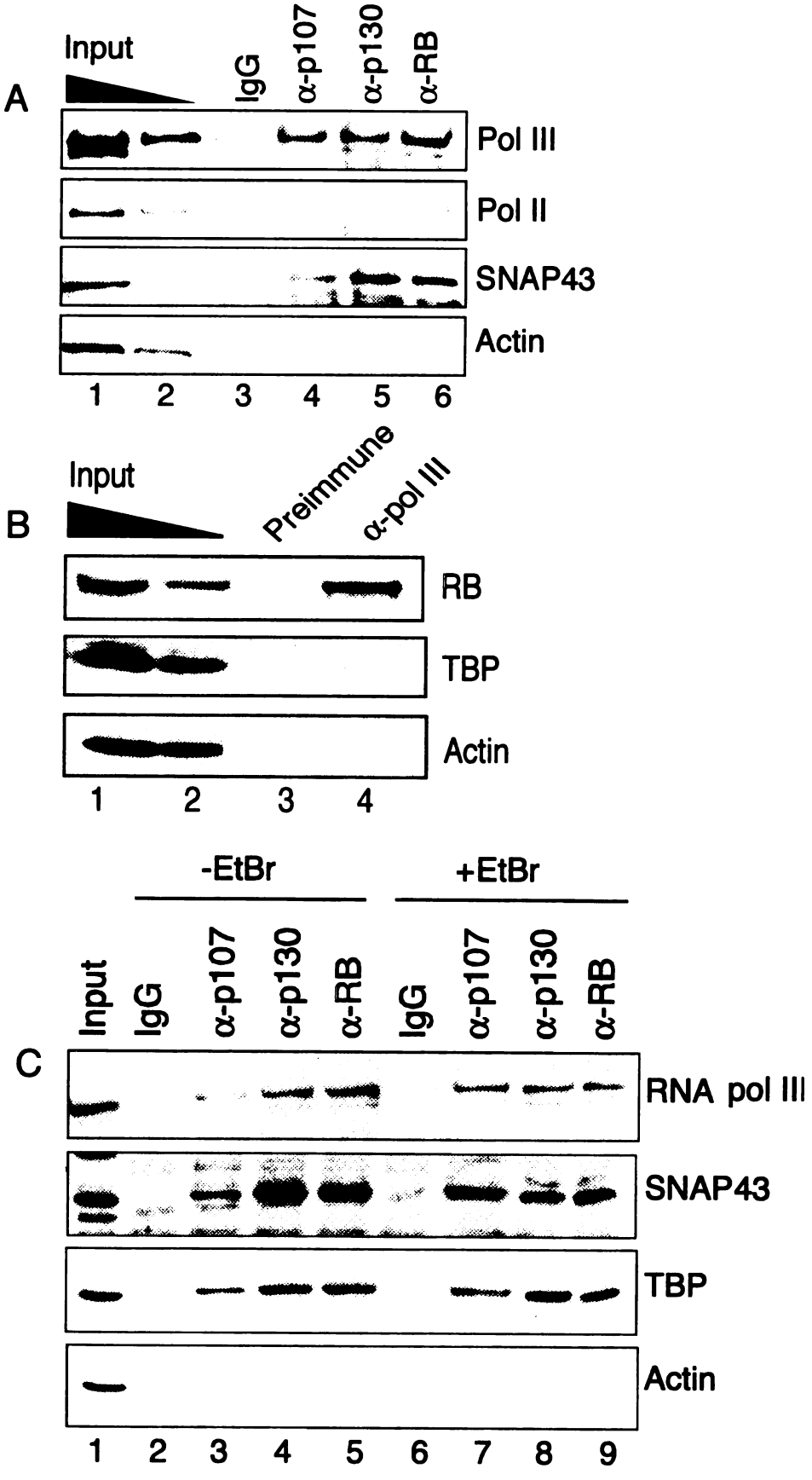
To test whether endogenous RB family proteins could bind to Pol III, and whether endogenous p107 and p130 also associate with SNAPc and TFIIIB, a co-immunoprecipitation assay was performed followed by Western blot analysis. HeLa cell nuclear extract was incubated with either goat IgG, anti-p107, anti-p130, or anti-RB antibodies for co-immunoprecipitation followed by Western blot analysis using either

anti-Pol III (largest subunit, RPC1), anti-Pol II (largest subunit, RPB1), anti-SNAP43, or anti-actin antibodies. As shown in Fig. 2-2 A, significant levels of Pol III (RPC1) and SNAP43 were detected in the samples immunoprecipitated with the anti-p107 (lane 4), anti-p130 (lane 5) and anti-RB (lane 6) antibodies but not with IgG (lane 3). However, Pol II was not detectable under the conditions used here for immunoprecipitation and Western blot analysis. As a non-specific co-immunoprecipitatin control, actin was not detected in any of those co-immunoprecipitation samples. Thus, all endogenous RB family members specifically associate with Pol III (RPC1) and SNAP43.

To further confirm the association between RB and Pol III (RPC1), a reciprocal co-immunoprecipitation assay and Western blot analysis was performed. HeLa cell nuclear extract was incubated with either preimmune or anti-Pol III (RPC1) serum for co-immunoprecipitation, followed by Western blot analysis using either anti-RB, anti-TBP or anti-actin antibodies. Detection of TBP was performed to determine whether TFIIIB mediates the association between RB and Pol III. As shown in Fig. 2-2 B, a significant level of endogenous RB was detected in the sample immunoprecipitated with anti-Pol III serum (lane 4) but not with the preimmune serum (lane 3). Neither TBP nor actin was detected in a significant level in any of the immunoprecipitated samples (lanes 3 and 4). The results shown by Fig. 2-2 B, not only confirm that endogenous RB associates with Pol III (RPC1), but also suggest that the association between RB family members and Pol III were unlikely mediated by TFIIIB complex, even though RB was found to interact with TFIIIB complex in previous research (26). p107 and p130 seemed also co-

Fig. 2-2. Endogenous RB family proteins associate with SNAP 43, TBP and Pol III. (A) Endogenous Pol III, SNAP43, but not Pol II and actin were co-immunoprecipitated with p107, p130 and RB. 600 µl of HeLa cell nuclear extract was incubated with 3 µg of p107 (lane 4), p130 (lane 5) or RB (lane 6) antibodies as well as with IgG antibody (lane 3). Protein-antibody complexes were precipitated by affinity purification using the protein G agarose beads (Upstate Inc.). The bound proteins were resolved by 7.5% SDS-PAGE, and the association of Pol III, Pol II, SNAP43 and actin with RB family proteins was detected by Western blot analysis. Lanes 1 and 2 show the relative amount of Pol III, Pol II, SNAP43 and actin present in 12 and 3 µl of nuclear extract. (B) Endogenous RB but not TBP or actin co-immunoprecipitated with Pol III. 600 µl of HeLa cell nuclear extract was incubated with rabbit preimmune (lane 3) and Pol III (largest subunit, RPC1) anti-serum (lane 4) antibodies. Subsequently, RB, TBP and actin antibodies were used for Western blotting analysis. Lanes 1 and 2 show the relative amount of RB, TBP and Actin present in the 30 and 12µl of HeLa cell nuclear extract. (C) The association of RB family proteins with Pol III (RPC1), SNAP43 and TBP is not DNA dependent. 600 µl of HeLa cell nuclear extract was immunoprecipitated with p107 (lanes 3 and 7), p130 (lanes 4 and 8) and RB (lanes 5 and 9) antibodies as well as goat IgG antibody (lanes 2 and 6) respectively. The reactions were carried out with (lanes 6-9) or without (lanes 2- 5) 100 µg/mL EtBr. Precipitated proteins were resolved by 7.5% SDS-PAGE, and the association of Pol III, SNAP43, TBP and actin with RB family proteins was assessed by Western blot analysis using the appropriate antibodies as indicated. Lane 1 shows the relative amount of Pol III (RPC1), SNAP43, TBP and actin present in 12 µl of HeLa cell nuclear extract.

Fig. 2-2


A
Input
IgG
α-p107
α-p130
α-RB
Pol III
Pol II
SNAP43
Actin
1 2 3 4 5 6
B
Input
Preimmune
α-pol III
RB
TBP
Actin
1 2 3 4
-EtBr
+EtBr
Input
IgG
α-p107
α-p130
α-RB
IgG
α-p107
α-p130
α-RB
C
RNA pol III
SNAP43
TBP
Actin
1 2 3 4 5 6 7 8 9

immunoprecipitated with Pol III (RPC1) (data not shown), further suggesting that all RB family proteins associate with Pol III (RPC1).

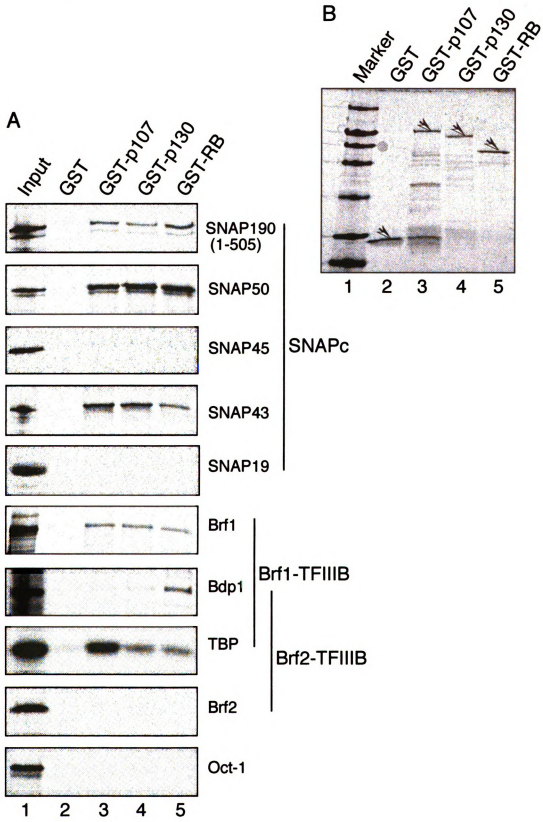
In order to ensure that the associations of RB family proteins and Pol III/SNAP43/TBP were not mediated by DNA, EtBr was added to the co-immunoprecipitation experiments to disrupt the normal DNA structure that is required for protein binding (18, 25). Two sets of co-immunoprecipitation assays were performed in parallel for comparison, one that was treated with EtBr and the other set that was not treated. Except for the EtBr treatment, all other operations of those two sets of co-immunoprecipitation assay were the same. Immunoprecipitated proteins were analyzed by Western blot, using anti-Pol III, anti-SNAP43, anti-TBP, and anti-actin antibodies. As shown in Fig. 2-2 C, Pol III (RPC1), SNAP43 and TBP were present in the immunoprecipitates of RB family proteins (lanes 3,4 and 5), suggesting that RB family proteins associate with Pol III (RPC1), SNAP43 and TBP. Furthermore, the co-immunoprecipitation levels of Pol III, SNAP43, TBP are comparable between the samples treated with (lanes 6,7,8 and 9) or without (lanes 2,3,4 and 5) EtBr, which indicates that the associations between RB family proteins and Pol III (RPC1), SNAP43 and TBP are not likely affected by the DNA present in the HeLa cell nuclear extract, at least in maintaining the association, though the DNA disruption by EtBr need to be tested in further.

RB family members can interact directly with multiple components of SNAPc and TFIIIB complexes

The results of previous co-immunoprecipitation assay showed that endogenous RB, p107 and p130 associate with SNAP43 and TBP, and the RB can interact directly with the subunits SNAP50 and SNAP43 of SNAPc, TBP, Brf1 and Bdp1 subunits of TFIIIB (13, 14). It is possible that p107 and p130 may associate with SNAPc or TFIIIB by interacting directly with subunit(s) belonging to SNAPc or TFIIIB. To test the possibility that p107 and p130 can interact directly with SNAPc and TFIIIB subunit(s), a GST-pull down assay with purified recombinant GST-p107 (249-1068) and GST-p130 (372-1139) was performed. The parallel GST pull-down assay with GST-RB (379-928) and GST were used as a reference and negative control, respectively. Each subunit of SNAPc, TFIIIB and Oct-1 was individually expressed in rabbit reticulocyte lysate system and labeled with ^{35}S-methione. Approximately, 1 μg of GST-p107 (249-1068), GST-p130 (372-1139), GST-RB (379-928), and GST was incubated with equivalent amount of each *in vitro* translated ^{35}S labeled protein. The levels of ^{35}S-labeled proteins recovered by GST fusion proteins were detected by autoradiography. As shown in Fig. 2-3 A, a significant amount of SNAP190 (1-505), SNAP50 and SNAP43 but neither SNAP45 nor SNAP19 of SNAPc were detected in samples treated with GST-p107 (249-1068) (lane 3), GST-p130 (372-1139) (lane 4) and GST-RB (379-928) (lane 5). For the TFIIIB complex, Brf1 and TBP were detected in all pull-downs with the GST tagged RB family proteins (lanes 3 to 5). Interestingly, another subunit of TFIIIB, Bdp1, was only present in the GST-RB (379-928) pull-down (lane 5). Neither Brf2, exists only in the TFIIIB used by U6 snRNA transcription, nor the U6 snRNA DSE binding protein, Oct-1, were detected in any pull down reactions (lanes 3 to 5). No association was detected with GST by any ^{35}S labeled proteins, suggesting that those subunits detected in the pull-down with the GST tagged

Fig. 2-3. All RB family proteins interact with multiple components of SNAPc and TFIIIB. (A) GST pull-down experiments were performed to test the interaction between RB family proteins and individual subunits of SNAPc and TFIIIB. Each SNAPc and TFIIIB subunit was expressed *in vitro* using rabbit reticulocyte lysates, and proteins were labeled with [^{35}S] methionine. The identities of SNAPc and TFIIIB are indicated. Lane 1 contains 10% of ^{35}S-labeled proteins used as an input. Interactions for [^{35}S] methionine labeled proteins were tested with GST (lane 2), GST-p107 (249-1068) (lane 3), GST-p130 (372-1139) (lane 4) and GST-RB (379-928) (lane 5). Proteins were separated by SDS-PAGE and visualized by autoradiography. (B) Equivalent mass of GST proteins was recovered by glutathione sepharose beads. 1μg each of GST (lane 2), GST-p107 (249-1068) (lane 3), GST-p130 (372-1139) (lane 4) as well as GST-RB (379-928) (lane 5) were prebound to glutathione sepharose beads. The proteins on beads were resolved by SDS-PAGE and visualized by Coomassie blue staining. Lane 1 contains protein molecular weight marker.

Fig. 2-3


A
Input
GST
GST-p107
GST-p130
GST-RB
SNAP190
(1-505)
SNAP50
SNAP45
SNAP43
SNAP19
SNAPc
Brf1
Bdp1
TBP
Brf1-TFIIIB
Brf2-TFIIIB
Brf2
Oct-1
1 2 3 4 5
B
Marker
GST
GST-p107
GST-p130
GST-RB
1 2 3 4 5

RB family proteins specifically interact with the RB family proteins. The results of Fig. 2-3 B shows that equal amounts of GST, GST-p107, GST-p130 and GST-RB were recovered during GST pull down processing. In summary, all RB family members may associate with SNAPc and TFIIIB through direct interacts with subunits within SNAPc and TFIIIB.

RNA polymerase III associates with the A domain of RB

RB has been shown to associate with SNAPc, TFIIIB and Pol III (RPC1). To understand the mechanism of how RB represses U6 snRNA transcription, it is important to identify the domain(s) of RB responsible for its association with those proteins. Previous work already identified that repression of U6 snRNA by RB requires the RB pocket A/B domain and C domain (14). Correspondingly, GST-RB containing the A/B and C domains (379-870) maintains the strong binding with SNAP43, SNAP50, TBP, Brf1 and Bdp1 (14). In contrast, GST-RB containing the pocket A/B domain (379-772) merely maintains strong interaction with SNAP50, and the GST-RB containing the A domain (379-577) does not bind to any one of those target proteins (14). To determine the regions of RB required for binding to Pol III, a series of truncated or mutated GST-RB fusion proteins were used in GST pull down assays. GST tagged proteins were incubated with HeLa cell nuclear extract. Proteins co-precipitated by GST tagged proteins were detected by Western blot analysis using anti-Pol III (largest subunit, RPC1), anti-Brg1, anti-actin, and anti-GST antibodies. Brg1 analyzed here served as a positive control, which was already known to associate with RB (7). The schematic representation of the various GST-RB proteins is shown in Fig. 2-4 A, and the GST pull-down results are shown in

Fig. 2-4. Pol III and Brg1 bind to the RB A domain. (A) Schematic representation of the truncated or mutated RB proteins used in the GST pull-down assay. (B) Characterization of the RB domains required for its associations with the RNA polymerase III and Brg1. GST pull down analysis was performed by incubating 300 µl of HeLa cell nuclear extract with 500 ng of GST (lane 2), GST-RB (379-928) (lane 3), GST-RB A/B/C domains (379-870) (lane 4), GST-RB A/B domain (379-772) (lane 5), GST-RB A domain (379-577) (lane 6), GST-RB B/C domains (645-870) (lane 7), GST-RB B domain (645-772) (lane 8), GST-RB Δexon22 (379-928) (lane 9) and GST-RB C706F (379-928) (lane 10). Precipitated proteins were resolved by 7.5% SDS-PAGE. The association of Pol III, Brg1 and actin with RB protein was assessed by Western blot analysis using Pol III (subunit RPC1) antiserum, Brg1 and actin antibodies. Lane 1 shows the relative amount of Pol III (RPC1), Brg1 and actin present in 15 µl of HeLa cell nuclear extract. Western blot analysis using GST antibody indicated the relative amount of GST tagged RB proteins recovered after processing.

Fig. 2-4

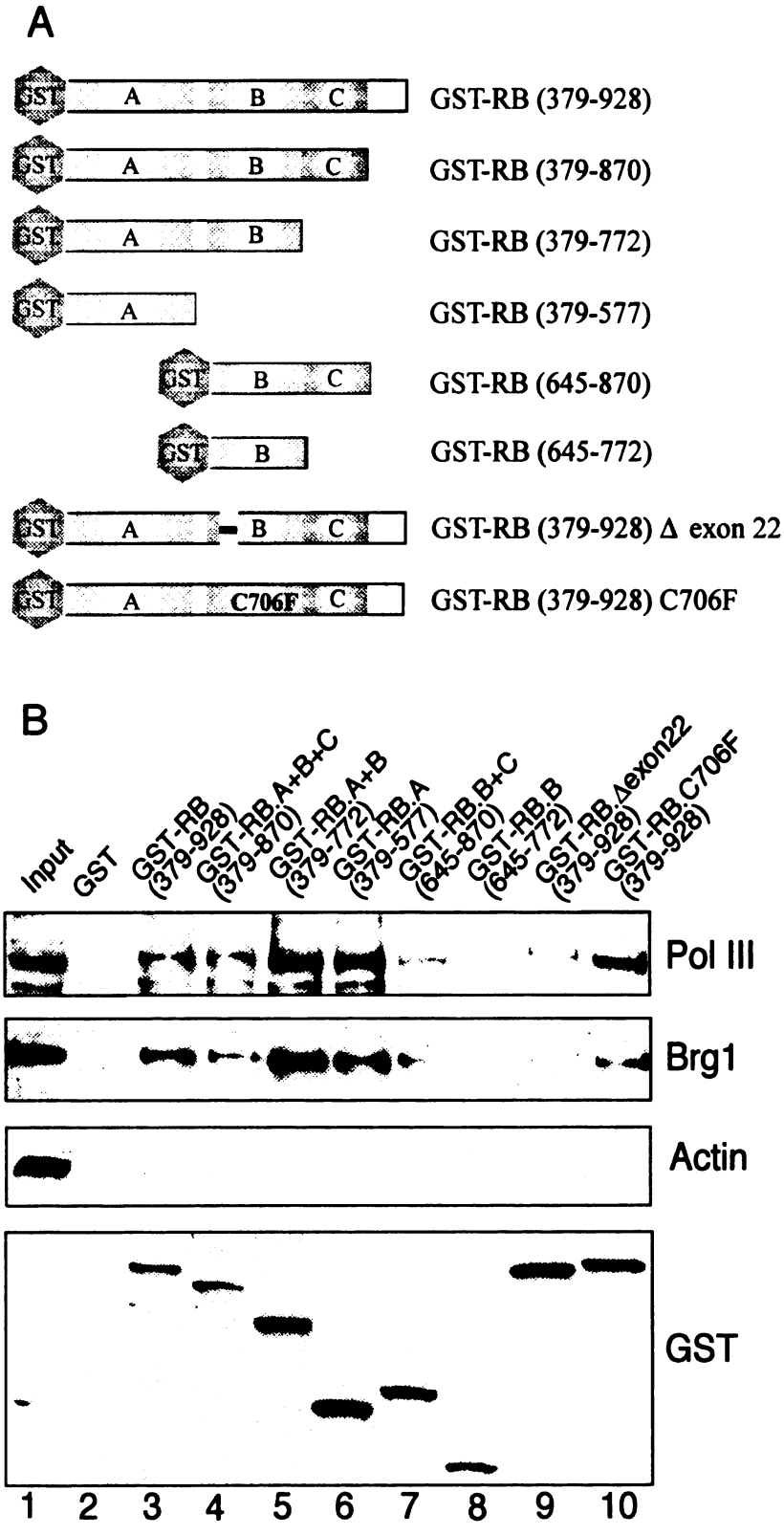

A
GST
A
B
C
GST-RB (379-928)
GST-RB (379-870)
GST-RB (379-772)
GST-RB (379-577)
GST-RB (645-870)
GST-RB (645-772)
GST-RB (379-928) Δ exon 22
C706F
GST-RB (379-928) C706F
B
Input
GST
GST-RB (379-928)
GST-RB A+B+C (379-870)
GST-RB A+B (379-772)
GST-RB A (379-577)
GST-RB B+C (645-870)
GST-RB B (645-772)
GST-RB Δexon22 (379-928)
GST-RB C706F (379-928)
Pol III
Brg1
Actin
GST
1 2 3 4 5 6 7 8 9 10

Fig. 2-4 B. Both Pol III and Brg1 were efficiently precipitated by GST-RB (379-928) (lane 3), GST-RB A/B/C domains (379-870) (lane 4), GST-RB A/B domain (379-772) (lane 5), GST-RB A domain (379-577) (lane 6), and GST-RB C706F (379-928) (lane 10), but not by GST-RB B/C domains (645-870) (lane 7), GST-RB B domain (645-772) (lane 8), and GST-RB Δexon 22 (379-928) (lane 9). Western blot analysis against GST indicated the relative amount of GST fused RB proteins recovered after GST-pull down processing. Conclusively, the A domain of RB is responsible for binding to Pol III (RPC1) as well as Brg1.

Discussion

The significance of RB family proteins in tumor suppression, cell-cycle progression control, cell differentiation and cell apoptosis is well established (5, 16, 17, 22). RB family proteins may also control cell growth by regulating the transcription of non-translated genes, such as ribosomal RNA, tRNA, and U6 snRNA that determine the protein synthesis rate (33). *In vitro* repression assays of Ad VA1 and U6 snRNA genes with recombinant RB family proteins (Fig. 2-1) supports the idea that all RB family members can repress Pol III transcription (27). Whether p107 and p130 are able to repress U6 snRNA transcription *in vivo* is yet to be investigated, but it is likely that p107 and p130 do repress U6 snRNA transcription *in vivo*, because endogenous p107 and p130 were found to occupy an endogenous U6 snRNA promoter (unpublished data by G. Jawdekar).

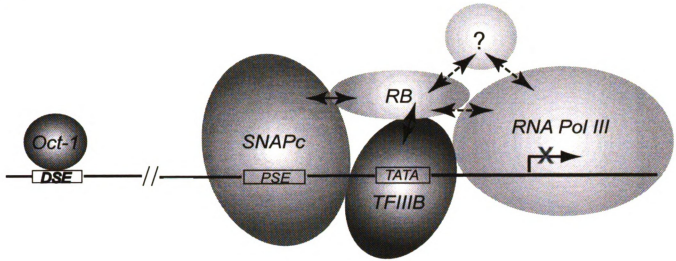
Pol III activity varies during the cell cycle progression, and has the highest activity during S and G2 phases but lowest activity during G1 and M phases (15, 34). RB family proteins may repress Pol III activity in a cell cycle dependent manner. RB and p130 target TFIIIB during G0 and G1 phases of cell cycle. RB is hypophosphorylated during G0 and G1 phase, and the hypophosphorylated RB binds to TFIIIB component, Brf1 (24). Whether RB family proteins target SNAPc in a cell cycle dependent manner is not known. All RB, p107 and p130 repress U6 snRNA transcription *in vitro* (Fig. 2-1), but whether they have overlapping or unique functions on U6 snRNA repression during cell cycle is not clear. RB, p107 and p130 may have some unique functions on U6 snRNA repression during cell cycle. Another possible distinction among RB family members on U6 snRNA repression is that different members of RB family may function in different tissues because of the discrete expression levels of RB family members among different tissues (29).

Except for the Bdp1subunit of TFIIIB, all subunits of SNAPc and TFIIIB that interact with RB also interact with p107 and p130 (Fig. 2-3). In addition, Pol III (RPC1) was found to associate with all RB, p107 and p130 (Fig. 2-2), and thus, it is likely that all RB family members share some common mechanism in repressing Pol III transcription. Bdp1 was detected to interact with RB only, and TBP was found to bind to p107 much stronger than RB and p130 (Fig. 2-3), however, the significance of these differences for RB, p107 and p130 to regulate the Pol III transcription is not known.

Previous findings in our lab demonstrated that the mechanism for RB to repress U6 snRNA transcription is distinct from the mechanism for tRNA repression (14). RB represses tRNA transcription by disrupting the preinitiation complex (PIC) formation at tRNA promoter (26). However RB does not disrupt the preinitiation complex (PIC) formation at the U6 snRNA promoter. The evidence for this idea is that RB, SNAPc, TFIIIB, and Pol III co-occupy the U6 snRNA promoter during repression (14). In the present work, RB was found to associate with multiple subunits of SNAPc, TFIIIB and Pol III. Although the integrity of SNAPc, TFIIIB and Pol III complexes is yet to be investigated, RB may function through a novel mechanism to repress the U6 snRNA. The idea is that RB tethers Pol III to SNAPc or TFIIIB and further prevents the Pol III from translocation that is required for transcription (Fig. 2-5). One other evidence for this model is that RB uses different regions to bind to SNAPc and TFIIIB than to Pol III. As identified here, the RB A domain is sufficient to associate with Pol III (Fig. 2-4). Though GST-RB Δexon 22 (379-928) has an intact A domain; it does not precipitate Pol III (RPC1) and Brg1 (lane 9 of Fig. 2-4). One possible reason is that internally truncated B domain influences the regular conformation of RB A domain. Previous findings indicate that RB binds subunits within SNAPc and TFIIIB using a region other than A domain (14), and the domain(s) in RB responsible for its binding to SNAPc and TFIIIB is discrete from the one binding to Pol III (RPC1). Whether RB interacts with Pol III directly or this interaction is mediated by other factor(s) is not known. Whether RB and Pol III association contributes to U6 snRNA repression is yet to be investigated. Additional data from our lab (unpublished data by T. Selvakumar) demonstrated that RB enriches the open complex on the U6 promoter, indicating RB tethers Pol III but does not

Fig. 2-5. A tether and immobilization model for RB repression of U6 snRNA transcription. RB simultaneously binds to Pol III and Pol III accessory factors including SNAPc and TFIIIB using discrete binding sites. One proposed consequence of RB binding is that RB prevents Pol III from translocation required for Pol III transcription. RB may bind to Pol III directly or indirectly through other unknown factor(s).

Fig. 2-5



cause Pol III to lose integrity. Since p107 and p130 were also demonstrated to associate with Pol III and multiple components of SNAPc and TFIIIB, it is possible that p107 and p130 also use a tether and immobilization model to repress U6 snRNA transcription.

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APPENDIX

Introduction

In eukaryotes genes are packaged into chromatin. Thus, histone modifications and changes in chromatin structure play a significant role in both positive and negative regulation of gene transcription. For example, histone acetylation levels which are controlled by histone acetyltransferases (HATs) and histone deacetylases (HDACs), significantly influence gene transcription (4). Another example for a role of histone modification in transcriptional regulation is histone methylation, which is controlled by methyl transferases (HMTs) and putative histone demethylase (4, 5). Chromatin structure can also be remodeled by the SWI/SNF complex and its effects on gene transcription can be either negative or positive (2). It is possible that histone modification and chromatin remodeling affect U6 snRNA transcription. As described by Zao *et al.* (2001), there is a nucleosome positioned between DSE and PSE sites of the U6 snRNA promoter, which are separated by approximately 150 bp. The positioned nucleosome mediates the coordinative binding between Oct-1 POU domain on the DSE and SNAPc on the PSE (6).

RB interacts extensively with HDACs, HMTs and the SWI/SNF subunits Brg-1/Brm (1), which are linked to histone modification and chromatin remodeling. RB induced histone modification or chromatin remodeling has been demonstrated to facilitate gene repression (1). Whether RB can repress U6 snRNA transcription by changing the structure of a nucleosome at the U6 snRNA promoter is unknown. To explore the role of chromatin structure in U6 snRNA transcription, a chromatinized U6 snRNA template was generated *in vitro*.

Materials and Methods

Preparation of *Drosophila* S-190 chromatin assembly extract

The preparation of *Drosophila* S-190 chromatin assembly extract followed the protocol created in Kadonaga laboratory (3). Approximately 80 g of *Drosophila melanogaster* embryos staged from 0 to 6 hours were collected. After extensive washing with water, embryos were dechorionated in 50% bleach for 90 seconds. Dechorionated embryos were cleaned by rinsing with Embryo Wash buffer (0.7% NaCl, 0.04% Triton X-100) and ddH_2O. Roughly cleaned embryos were moved to a glass beaker and further washed 2 times with cold (4°C) Embryo Wash buffer to remove chorion particles. Trace Embryo Wash buffer left in the cleaned embryos was washed out with Saline Wash (0.7% NaCl), and then trace Saline Wash buffer was further washed once with Buffer R (1.5 mM MgCl2, 1 mM DTT, 10 mM KCl, 10 mM β-glycerophosphate, 0.5 mM EGTA, 10% glycerol, 10 mM HEPES, pH 7.5, 0.2 mM PMSF). Buffer R was then added to double volume of settled embryos. Embryos in Buffer R were homogenized in a 40 mL Wheaton Dounce Homogenizer until no intact embryos were left. Embryo homogenates were then centrifuged at 7,650 X g for 5 min at 4°C. After the centrifugation, the golden brown supernatant between the top foamy layer and bottom pellet was transferred to a new tube using a syringe with 18-gauge needle. $MgCl_2$ was added to give a final concentration of 7 mM, and then the supernatants was clarified through centrifugation in an SW41 rotor (Beckman Co.) at 40,000 rpm (198,000 X g) for 135 min at 4°C. After centrifuge clarification, a middle liquid layer with yellow brown color, which is the S-190 chromatin assembly extract, was collected and frozen quickly in liquid nitrogen and

placed in -80°C storage. Pre-clarified S-190 was clarified one more time through centrifugation in a SW50.1 rotor at 45,600 rpm (192,000 X g) for 135 min at 4°C before S-190 was used to assemble chromatin.

Preparation of *Drosophila* histones

a) Preparation of embryo nuclei

150 g of *Drosophila melanogaster* embryos with age from 0 to 12 hours were collected and dechorionated with 50% bleach for 90 seconds. After dechorionation, embryos were extensively rinsed with Embryo Wash buffer and ddH_2O. The dechorionated embryos were weighed and suspended in 3 mL of Buffer B (0.5 mM EGTA, 2 mM EDTA, prepared in Buffer A with 15 mM Tris, pH 7.5, 0.1% (v/v) 2-mercaptoethanol, 0.34 M sucrose, 0.15 mM spermine, 15 mM NaCl, 0.5 mM spermidine, 60 mM KCl, 0.25 mM PMSF) per gram of embryos. Embryos in Buffer B were homogenized using a Yamato homogenizer. The homogenate was filtered through miracloth (Calbiochem) into Sorvall GSA rotor bottles to remove debris. Homogenate was subjected to centrifugation at 8,000 rpm (10,400 X g) for 20 min at 4°C to precipitate nuclei. After centrifugation, the loose nuclei pellet in the tubes was transferred to another centrifuge tube and suspended in 200 mL of Buffer A. Homogenate in Buffer A was centrifuged again at 8,000 rpm (10,400 X g) for 10 min at 4°C. The nuclei pellet in the tube was collected for second wash. Finally, nuclei pellet was suspended in 30 mL of Buffer A to obtain a DNA and protein concentration of 100 Abs_{260} units/mL.

b) Test micrococcal nuclease (MNase) digestion

1 mL of embryo nuclei suspended in Buffer A was transferred to 1.5 mL microcentrifuge tubes at 37°C and added with $CaCl_2$ to a final concentration of 1mM. The genomic DNA was digested with 0.4 units of MNase. Digestion continued for 1, 2, 4, 6, 8, 10, 15, or 20 minutes. Following this, 100 µl of nuclei was transferred to another tube containing 2.5 µl of 0.5 M EDTA to quench the MNase digestion. Then, the genomic DNA from each sample was recovered with chloroform treatment and ethanol precipitation. The extent of DNA digestion was analyzed on a 1.5% agarose gel.

c) Bulk digestion of nuclei by MNase and purification of histones.

Bulk nuclei were digested for the time determined to get most 10-nucleosome fragments in the test digestion and then stopped by 10 µM EDTA. After digestion, the nuclei were precipitated by centrifugation at 10,000 rpm in an SS34 rotor (12,000 X g) for 10 min at 4°C. The precipitated nuclei were subjected to lysis in 10 mL of 10 mM EDTA and 0.5 M NaCl. Then the lysed nuclei were clarified by centrifugation at 10,000 rpm in an SS34 rotor (12,000 X g) for 5 min at 4°C. Supernatant was collected, followed by measurement of DNA absorbance (Abs_{260}). Histones in the supernatant were separated in Beckman SW28 rotor tubes with 36 mL of 5% to 30% linear sucrose gradient. Each sucrose gradient was loaded with lysis supernatant of 500 Abs_{260} units or less. Then the loaded sucrose gradients were centrifuged at 26,000 rpm in an SW28 rotor (89,500 X g) for 16 hr at 4°C. After centrifugation, the sucrose gradient was fractionated to 1.1 mL per fraction at 4°C. Histones in each fraction from a representative gradient were checked by electrophoresis on a 15% SDS-polyacrylamide gel and visualized by Coomassie blue staining. Peak fractions of core histones were pooled together in dialysis tubing and then

dialyzed overnight against 4 liters of Tris/EDTA solution (4 mM EDTA, 50 mM Tris, pH 7.9). The genomic DNA in histones was removed by passing through hydroxyapatite column. The amount of DNA in the dialyzed histones was determined upon Abs_{260}. The volume of hydroxyapatite column was prepared according to 1 mL of hydroxyapatite per 1.5 mg DNA. The preparation of hydroxyapatite column, as well as the sample loading, washing, and fractionating were facilitated by an AKTAexplorer Chromatography apparatus. Hydroxyapatite column was equilibrated in HC Buffer (1 mM DTT, 0.2 mM PMSF, 40 mM Na_2HPO_4, pH 6.8) before loading the sample. After loading the sample, the hydroxyapatite column was washed with 3 to 4 column volumes of HC Buffer/0.35 M NaCl. Core histones were eluted from the hydroxyapatite column with HC Buffer/2.5 M NaCl. The eluted core histones were collected as 1 mL fractions. Histones in each fraction were checked by electrophoresis in a 15% SDS-polyacrylamide gel and Coomassie blue staining visualization. The fractions with peak core histones were pooled together, and the DNA in these fractions was analyzed on 1% agarose gel. The core histones clarified from DNA were dialyzed overnight at 4°C against 4 liters of Core Histone Storage buffer (10% glycerol, 1 mM EDTA, 10 mM KCl, 10 mM HEPES, pH7.6). Finally, the concentration of core histones was measured by using Bradford Protein Assay (Bio-Rad). The histone H1 preparation followed the same procedure as used for core histones.

In vitro chromatin assembly

Chromatin assembly on U6 template, pU6/Hae/Ra.2, followed the protocols as described in previous papers (3, 6). To start chromatin assembly on 1μg of pU6/Hae/Ra.2 template,

50 µl of *Drosophila* S-190 chromatin assembly extract was mixed with 0.96 µg of core histones and 0.9 µg of histone H1 (optional) in Buffer R (1.5 mM $MgCl_2$, 1 mM DTT, 10 mM KCl, 10 mM β-glycerophosphate, 0.5 mM EGTA, 10% glycerol, 10 mM HEPES, pH 7.5, 0.2 mM PMSF) to a final volume of 170 µl, followed with incubation at room temperature for 30 minutes. After incubation, 28.5 µl of ATP mix (42.5 µl of 0.5 M phosphocreatine, 4.25 µl of 0.5 M ATP, 22.7 µl of ddH_2O, 29.7 µl of 0.1 M $MgCl_2$, 0.84 µl of creatine phosphokinase solution) was added. Creatine phosphokinase solution was composed of 50 mM NaCl, 10 mM potassium phosphate with pH 7.0, 50% (v/v) glycerol, 5 mg/mL creatine phosphokinase of type I (Sigma). Then 0.32 µg of Oct-1 and 1 µg of pU6/Hae/Ra.2 were added. Finally, the chromatin assembly reactions were incubated at 27°C for 5 hours to complete assembly.

MNase digestion to detect chromatin assembly on pU6/Hae/Ra.2

50 µl of assembled chromatin was transferred to each of four 1.5 mL microcentrifuge tubes containing 1.5 µl of 0.1 M $CaCl_2$. 5 µl of the MNase solutions with different concentrations, 1.3 units/mL, 4 units/mL, 12 units/mL and 36 units/mL was added respectively to the tubes to start digestion at room temperature for 10 minutes. Then the digestion was stopped by adding 5 µl of MNase stop buffer (0.24 mM EDTA, 29 µg/mL RNase). The digestion samples were treated with 100 µl of 0.125 mg/mL Proteinase K solution prepared in transcription stop solution (1% SDS, 0.2 M NaCl, 0.25 mg/mL glycogen Type IX, 20 mM EDTA, pH 8.0). DNA in the digestion samples was recovered by phenol extraction and ethanol precipitation. The precipitated DNA fragments were separated by electrophoresis on a 1.25 % agarose gel for EtBr visualization.

Results

Histone preparation

Nuclei for histone preparation were prepared from 150 g of *Drosophila melanogaster* embryos with age from 0 to 12 hours. The optimal time of MNase digestion was measured in a test digestion. As shown in Fig. A-1, the digestion for 4 minutes produced most DNA fragments of 10 nucleosomes in length. This length of chromatin provides adequate separation from contaminating proteins during the subsequent gradient treatment. After the determination of the optimal digestion time, nuclei were subjected to bulk digestion. Nuclei were then lysed to release the histones. Histones were separated by linear sucrose gradients. After separation, the gradients were fractionated in 1.1 mL fractions. The histone distribution in fractions from a representative sucrose gradient (No.1) was tested on a 15% SDS-PAGE. As shown in Fig. A-2, pure core histones distributed only from fraction 1 to fraction 22. Histone H1 appeared from fraction 23 to fraction 35. Two fractions, 15 and 25th, from another gradient (No.5) demonstrated similar histone distribution. The peak core histones were pooled together (pool 3 in Fig. A-2) for further histone concentration and DNA removal by passing through hydroxyapatite column. After chromatography process, histone distribution and genomic DNA in the fractions from flowthrough, wash, and elution fractions were analyzed on a SDS-PAGE and agarose gel, respectively. As shown in Fig. A-3, Core histones were concentrated in 3 fractions: 15, 16 and 17th (Fig. A-3). As shown in Fig. A-4, there is no visible DNA in any samples collected after passing through hydroxyapatite column. In contrast, a DNA ladder in the sample from pool 3 before passing through hydroxyapatite column was observed, suggesting that the concentrated histones are free of DNA. The

Fig. A-1. Optimization of Micrococcal Nuclease (MNase) digestion. DNA ladders were made after nuclei were partially digested by MNase for varying time points as indicated (see materials and methods). MNase digested nuclei were separated by 1.5% agarose gel electrophoresis and visualized by EtBr staining. Nuclei digested for 4 minutes were used for following histone preparation.

Fig. A-2. Histone distribution among fractions after fractionated from sucrose gradients. 40 μl of each selected fraction was boiled with 10 ul 5X laemli buffer. Histones in each sample were separated by 15% SDS-PAGE and then visualized by Coomassie blue staining. Chicken total histones were used as an indicator. Pool 3 with peak core histones was used for the core histone preparation. Pool 5 with peak histone H1 was used for the histone H1 preparation.

Fig. A-1

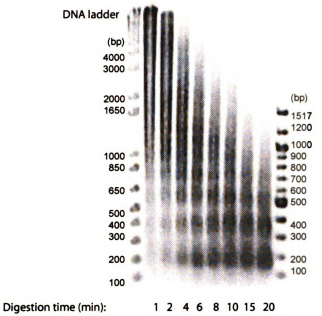
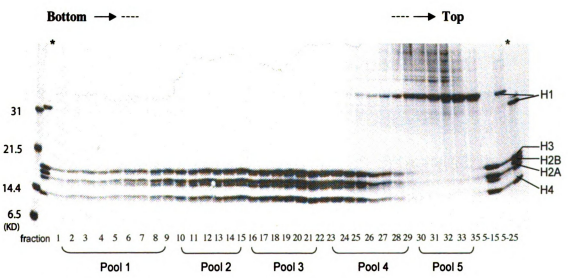


Fig. A-2



* Chicken histones

Fig. A-3. Core histones were concentrated by hydroxyapatite column chromatography. 20 μl of each representative sample from flow through, wash and fractionation was boiled with 5 μl of 5 X Laemmli buffer. Core histones in each sample were separated by 15% SDS-PAGE and then visualized by Coomassie blue staining. Lane "P3" is core histones before passing through hydroxyapatite column. Lane "*" is the chicken histones as an indicator of molecular weight.

Fig. A-4. Genomic DNA was depleted from core histones by hydroxyapatite column chromatography. 20 μl of each representative sample from flow through, wash and fractionation was resolved by 1% agarose gel and then visualized by EtBr staining. Lane "P3" indicates the genomic DNA in the core histones before passing through the hydroxyapatite column.

Fig. A-3

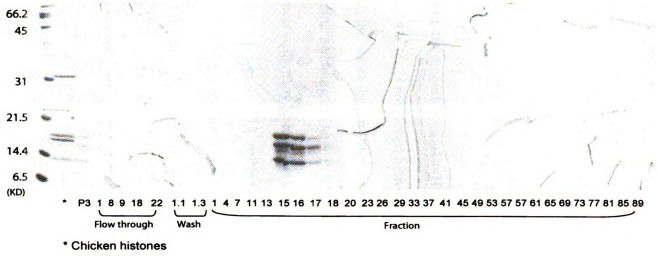
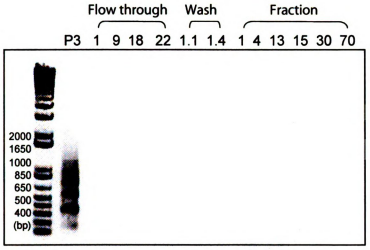

66.2
45
31
21.5
14.4
6.5
(KD)
* P3 1 8 9 18 22 1.1 1.3 1 4 7 11 13 15 16 17 18 20 23 26 29 33 37 41 45 49 53 57 57 61 65 69 73 77 81 85 89
Flow through
Wash
Fraction
* Chicken histones

Fig. A-4


Flow through
Wash
Fraction
P3 1 9 18 22 1.1 1.4 1 4 13 15 30 70
2000
1650
1000
850
650
500
400
(bp)

fractions with concentrated core histones were pooled together for dialysis against storage buffer. Finally, the concentration of total core histones was measured by Bradford assay. The core histone concentration was 1 μg/μl. The histone H1 preparation followed the same procedure used for core histone preparation, except using the pool 5 (Fig. A-2). The concentration of purified histone H1 was 2 μg/μl.

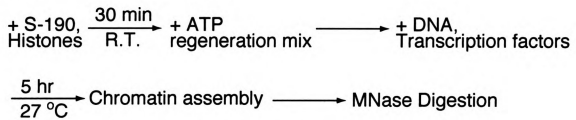
Chromatin assembly on pU6/Hae/Ra.2 template

A nucleosome is positioned between the PSE and the DSE on U6 snRNA promoter, which facilitates the U6 snRNA transcription. The positioned nucleosome mediates a coordinate binding of Oct-1 POU domain and SNAPc on the U6 promoter (6). RB interacts with many histone modifiers and chromatin remodeling factors (1). To test whether RB represses U6 snRNA transcription by affecting the nucleosome structure on the U6 snRNA promoter, it is necessary to obtain an *in vitro* chromatinized U6 snRNA template. Chromatin assembly on a U6 snRNA template was performed as described in Materials and Methods. The quality of *in vitro* chromatinized template was checked by partial MNase digestion and agarose gel electrophoresis. As shown in Fig. A-5 B, regularly spaced DNA ladders were seen in all sets of chromatin assembly upon MNase digestion. Chromatin assembled with core histone only showed a DNA ladder with about 160 bp spacing between two consecutive bands (Fig. A-5 B), reflecting the length of DNA fragments surrounding the core nucleosome. Whereas, a DNA ladder with about 200 bp spacing between two consecutive bands was displayed in assembled samples containing both core histones and histone H1 (Fig. A-5 B), reflecting the length of DNA

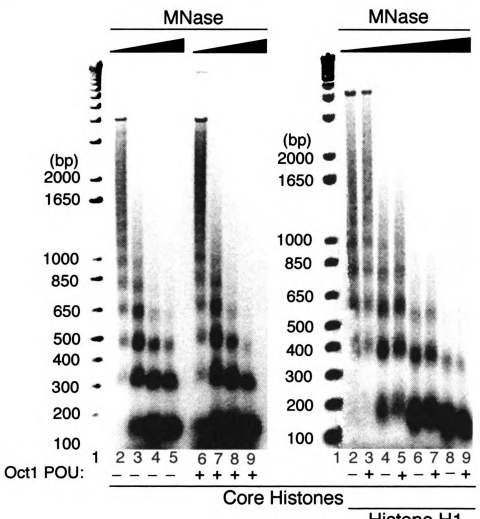
Fig. A-5. Chromatin was assembled on pU6/Hae/Ra.2 and the Oct-1 POU domain did not interrupt the overall chromatin assembly. (A) Schematic representation of the procedure of chromatin assembly (see materials and methods). (B) The assembled chromatin was partially digested by Micrococcal Nuclease (MNase) with different concentrations: 1.3, 4, 12 and 36 U/mL, for 5 minutes. After digestion, the DNA fragments were separated on a 1.25% agarose gel and visualized by EtBr staining. In the left panel, chromatin was assembled by core histones alone, with (lanes 6-9) or without (lanes 2-5) Oct-1 POU domain. In the right panel, chromatin was assembled by core histones and histone H1, with (lanes 3,5,7 and 9) or without (lanes 2, 4, 6 and 8) Oct-1 POU domain.

Fig. A-5

A


+ S-190,
Histones
30 min
R.T.
+ ATP
regeneration mix
+ DNA,
Transcription factors
5 hr
27 °C
Chromatin assembly
MNase Digestion

B


MNase
MNase
(bp)
2000
1650
1000
850
650
500
400
300
200
100
1 2 3 4 5 6 7 8 9
Oct1 POU: – – – – – + + + +
1 2 3 4 5 6 7 8 9
– + – + – + – + –
Core Histones
Histone H1

fragments surrounding the core nucleosome plus the linker DNA. Comparing the DNA ladder between samples with the Oct-1 POU domain (Fig. A-5 B left panel lanes 6 to 9, right panel lanes 3,5,7and 9) and samples without the Oct-1 POU domain (Fig. A-5 B left panel lanes 2 to 5, right panel lanes 2,4,6 and 8), the length of DNA fragment contained in a nucleosome was not changed by the Oct-1 POU domain. This suggests that the transcription factor Oct-1 POU domain does not interrupt the overall chromatin assembly on pU6/Hae/Ra.2, which is consistent with the previous report (6). The chromatinized pU6/Hae/Ra.2 will be used in future transcriptional assays.