RESEARCH COMMUNICATION

Active repression and E2F inhibition by pRB are biochemically distinguishable

John F. Ross,^{1,3} Anders Näär,² Hieu Cam,¹ Richard Gregory,¹ and Brian David Dynlacht^{1,4}

¹Harvard University, Department of Molecular and Cellular Biology, Cambridge, Massachusetts 02138, USA; ²Howard Hughes Medical Institute, Department of Molecular and Cellular Biology, University of California, Berkeley, California 94720, USA

To understand mechanistically how pRB represses transcription, we used a reconstituted transcription assay and compared pRB activity on naked versus chromatin templates. Surprisingly, when pRB was directly recruited to a naked template, no transcriptional repression was observed. However, we observed active repression when the same promoter was assembled into chromatin. Histone deacetylases do not appear to play a role in this observed repression. Further experiments showed repression could occur after preinitiation complex assembly, in contrast with pRB inhibition of E2F, suggesting discrete mechanisms by which pRB directly inhibits an activator such as E2F or actively represses proximally bound transcription factors.

Received October 11, 2000; revised version accepted December 27, 2000.

The retinoblastoma tumor suppressor protein (pRB) modulates cell cycle progression through transcriptional regulation of the genes required for the G₁ to S transition (for review, see Dyson 1998). Different modes of activity of pRB have been observed. For example, pRB can directly bind to and inactivate certain promoter-bound transcription factors, the most notable of which is E2F. We have shown previously that pRB can inhibit E2F in vitro on naked promoter templates without a requirement for chromatin (Dynlacht et al. 1994; Ross et al. 1999). Other experiments in which pRB was recruited directly to a promoter by fusion to a DNA-binding domain show that pRB also can repress transcription independent of recruitment by a promoter-bound transcription factor such as E2F (Adnane et al. 1995; Bremner et al. 1995; Sellers et al. 1995; Weintraub et al. 1995; Zhang et al. 1999). This latter activity, termed active repression (or transrepression), can be invoked to explain transcriptional repression of activator proteins bound to the proximal promoter.

[Key Words: pRB; E2F; transcriptional repression; chromatin]

³Present address: Genetics Institute, Cambridge, MA 02140, USA.

⁴Corresponding author.

E-MAIL dynlacht@biosun.harvard.edu; FAX (617) 496-1391.

Article and publication are at www.genesdev.org/cgi/doi/10.1101/gad.858501.

Recent studies have implicated histone deacetylases (HDACs) and the human SWI-SNF complex as required components for pRB repression in certain situations (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998; Zhang et al. 2000). Here, pRB is thought to recruit HDAC and SWI-SNF to the promoter, whereupon these enzymes alter the chromatin structure to that of a repressed state. Interestingly, the requirement for HDAC activity appears to be limited to a subset of promoters, and recent in vivo experiments suggest that repression by pRB occurs through both HDAC-dependent and -independent mechanisms (Luo et al. 1998; Chen and Wang 2000; Dahiya et al. 2000). Two other proteins, CtBP and RBP1, have been implicated as pRB-dependent transcriptional corepressors of E2F activation (Lai et al. 1999; Meloni et al. 1999).

Nevertheless, the detailed mechanisms by which pRB selectively represses transcription have not been fully elucidated, in part because of the lack of a simplified in vitro system able to recapitulate active repression. Recent studies have provided much insight into the factors involved in the assembly of chromatin and the regulation of transcription from such templates (Workman and Kingston 1998; Vignali et al. 2000). We have taken advantage of these tools to reconstitute active repression by pRB in vitro and to determine whether this mechanism is related to inhibition of the E2F transcription factor. Using an in vitro transcription assay with purified components, we compared the requirements for active repression with inhibition of E2F on both naked and chromatinized templates. These experiments and order of assembly assays indicated that active repression by pRB required the assembly of a responsive promoter into chromatin whereas inhibition of E2F did not. Although active repression required a chromatin template, histone deacetylase activity was not necessary for transcriptional repression. Our results clearly delineate distinct mechanisms whereby active repression and inhibition of E2F by pRB are accomplished by targeting unique sets of transcription factors.

Results

To understand how pRB can actively repress transcription without prior recruitment of E2F, we expressed a fusion protein in which the Gal4 DNA-binding domain was fused to the amino terminus of full-length pRB. Expression of a similar fusion protein has been used previously in transfection assays to recruit pRB directly to a linked promoter, without the need for prior binding by E2F (Adnane et al. 1995). We were able to use a transient expression approach to confirm that Gal4-RB can potentially repress a promoter containing both Gal4 and Sp1 sites (Fig. 1A). As expected, neither expression of excessive amounts of the Gal4 DNA-binding domain nor pRB (relative to Gal4-RB) resulted in repression of this promoter. In this setting, repression by pRB did not require

Figure 1. Gal4-RB directly represses transcription in vivo and retains the ability to inhibit E2F in vitro. (A) Human pRB-deficient C33A cells were transiently cotransfected with the pG₅Sp1E4-CAT reporter containing Gal4 and Sp1 binding sites and the indicated plasmids, and CAT assays were performed as described in Materials and Methods. Either no pRB or increasing amounts of pGal4-RB (+, ++, +++, and ++++ indicate 0.25, 1.25, 2.5, and 5 μ g of vector, respectively) were included. As controls, full-length pRB (1.25 μ g pCMV-RB) and the Gal4 DNA-binding domain (5 μ g pSG42Y) also were tested. Representative data from three independent experiments are shown. (B) Silver-stained SDS-polyacrylamide gels of representative purified factors used in the in vitro transcription reactions. (C) In vitro transcription using p(E2F)₄B-Gless as a template. E2F, Gal4-RB, and pRB were included as indicated.

recruitment of HDACs, because treatment of transfected cells with the HDAC inhibitor trichostatin A (TSA) did not alleviate transcriptional inhibition (data not shown).

To begin characterizing this activity in vitro, we expressed and purified recombinant Gal4-RB and all other factors required to reconstitute transcriptional activation (Fig. 1B). To verify that recombinant Gal4-RB protein retained the previously documented E2F inhibitory activity in vitro, we conducted in vitro transcription assays by using a naked template and a simple E2F responsive promoter containing multimerized binding sites. We tested E2F-4/DP-1 complexes in the absence and presence of pRB because the E2F-4/DP-1/pRB complex represents the largest portion of nuclear E2F repressor complexes in growing cells (J. Lees, pers. comm.). We observed robust activation in the presence of purified E2F-4/DP-1 heterodimers (we refer to the E2F4/DP1 heterodimer simply as E2F throughout this article) and potent repression by Gal4-RB (Fig. 1C). Indeed, its ability to inhibit E2F was indistinguishable from pRB lacking Gal4 moieties.

To test the ability of Gal4-RB to repress Sp1 mediated transactivation (through active repression) in vitro, we linked the same promoter containing Gal4 sites and Sp1 sites used in our transient transfection assays to a G-less cassette reporter and performed reconstituted transcription assays. Surprisingly, we were unable to observe repression of Sp1 activation by Gal4-RB by using our highly purified basal transcription factors and a naked template (Fig. 2A, lanes 1–7). To determine if the purified general transcription factors were missing a required cofactor, we replaced our purified factors with less pure preparations, including a crude nuclear extract. In each case, we were unable to reconstitute repression of Sp1 by Gal4-RB (data not shown).

Because recent evidence shows that in some cases pRB may function through interactions with proteins that modulate chromatin structure, we assembled our Sp1 responsive promoter into chromatin and tested the effect of Gal4-RB. Here, chromatin assembly was performed using a *Drosophila* embryo S190 extract and purified histones. We verified that this template and our E2F-responsive promoter were packaged into physiologically spaced nucleosomal arrays by using micrococcal nuclease assays (Fig. 3B; data not shown). Furthermore, activation by Sp1 was completely dependent on addition of the chromatin-specific coactivator ARC (activator recruited cofactor; Näär et al. 1999) when ARC was immunodepleted from our system, again confirming the efficiency of our chromatin assembly reactions (data not shown). Remarkably, Gal4-RB efficiently repressed transcription under

these conditions (Fig. 2A, lanes 8–14). Repression was specific, because neither wild-type pRB nor the Gal4 DNA-binding domain alone diminished transcription (lanes 10 and 11). This activity of pRB appears to be fundamentally different from the ability of pRB to inhibit E2F, given that inhibition of E2F did not require chromatin assembly. Indeed, pRB and Gal4-RB could inhibit activation by E2F to a similar extent on both naked and chromatinized templates (Fig. 2B; data not shown).

To verify that the observed Gal4-RB-mediated repression was not due to inhibition of Sp1 binding to DNA, we developed an in vitro chromatin immunoprecipitation (ChIP) assay. Using conditions identical to our reconstituted transcription reactions, we assembled chromatin and incubated the resulting templates with Sp1, Gal4-RB, or both. As a control, mock assembly reactions were performed in the absence of plasmid DNA. After cross-linking and immunoprecipitation of Sp1, we detected robust levels of Gal4-RB in a template-dependent manner (Fig. 2C). Therefore, Gal4-RB and Sp1 can simultaneously bind the chromatinized promoter template, and repression does not occur by simply suppressing Sp1 binding.

Because the above chromatin assembly reactions relied on a crude (and therefore less defined) S190 extract, we tested Gal4-RB activity on chromatin assembled using highly purified recombinant factors. Here, we replaced the S190 extract with dNAP1 and ACF, as Ito et al. (1999) have shown recently that these factors, together with histones, can assemble regularly spaced nucleosomes. Recombinant dNAP1 and the two subunits of ACF (ACF1 and ISWI) were purified to near homogeneity from virally infected insect cell extracts (Fig. 3A). Chromatin assembled with the S190 extract as well as the recombinant ACF and subjected to micrococcal nuclease digestion produced a ladder of bands indicative

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Figure 2. Gal4-RB can repress transcription in vitro on a chromatinized but not a naked template, whereas pRB can inhibit E2F similarly on either template. (A) In vitro transcription using pG₅Sp1E4-Gless as a template. Transcription was performed using the indicated factors on a naked (lanes 1–7) or chromatin (lanes 8–14) template. Five nanograms of Sp1, 20 ng of pRB, 5 ng of Gal4, and 5, 12.5, and 25 ng of Gal4-RB were used per reaction as indicated. The chromatin template was assembled using *Drosophila* S190 extract. The numbers beneath the lanes represent quantitated relative transcription levels. Basal transcription levels for each panel (lanes 1 and 8) were given unit value. Quantitation was performed as described in Materials and Methods. (B) Transcription reactions were performed as in A, except that reactions included the p(E2F)₄B-Gless template, E2F-4/DP-1 (12.5 ng of each subunit per reaction), and 10 ng Gal4-RB. Note that transcription using chromatin results in a lower signal than that of a naked template; hence, autoradiograms displaying transcription from chromatin templates typically required five times longer exposure. (C) In vitro chromatin immunoprecipitations indicate that Gal4-RB does not preclude DNA binding by Sp1. Chromatin was reconstituted with pG₅Sp1E4-Gless as a template or mock reconstituted without DNA and incubated with Flag-tagged Sp1, Gal4-RB, or both as indicated above the panel. Proteins and DNA were cross-linked and immunoprecipitated with anti-Flag antibodies as described in Materials and Methods. After reversal of cross-links, samples were electrophoresed and Western blotted sequentially with anti-pRB and anti-Flag antibodies (to detect Sp1). Western blotting with antibodies against Gal4-RB and Sp1 also confirmed that each was present in the indicated reactions before immunoprecipitation (input; data not shown).

of physiologically spaced nucleosomes, indicating that both systems were comparably efficient at assembling nucleosomes into regularly spaced arrays (Fig. 3B). When we tested this chromatinized Sp1 responsive promoter assembled with recombinant factors as a template for transcription, we observed both Sp1 activation and robust repression by Gal4-RB, whereas inclusion of pRB lacking Gal4 residues resulted in a minimal decrease in activity (Fig. 3C). Therefore, using two independent chromatin assembly systems, Gal4-RB was able to completely repress Sp1-activated transcription, although it had no discernible effect on the corresponding naked template.

To further characterize the repressive effect of RB on a chromatin template, we conducted order of addition experiments. Our previous experiments using naked templates indicated that E2F-responsive promoters containing a preassembled preinitiation complex (PIC) were refractory to pRB repression of E2F, suggesting that E2F recruitment of the PIC prevented subsequent pRB associa-

tion and function (Ross et al. 1999). Therefore, to further compare and contrast pRB inhibition of E2F activation with active repression of chromatin, we incubated Gal4-RB with a chromatin template on which Sp1 and the preinitiation complex had been assembled previously (Fig. 4A). Interestingly, Gal4-RB was able to completely repress Sp1-activated transcription regardless of whether Gal4-RB was added before or after PIC assembly (Fig. 4B). Importantly, in parallel studies, Gal4-RB was capable of repressing E2F activation only if the protein were present before or during PIC assembly (Fig. 4C). These results confirm that active repression by pRB occurs by a mechanism fundamentally different from direct inhibition of E2F. Recent experiments have suggested that pRB may function by recruiting HDACs and that HDAC activity is responsible for establishing a repressed chromatin conformation (Brehm et al. 1998; Luo et al. 1998; Magnaghi-Jaulin et al. 1998). When the HDAC inhibitor TSA was tested in a purified transcription system with the HIV-LTR template and purified p65/50 and Sp1, modest (twofold) derepression of the promoter was observed (A.M. Näär and P. Beaurang, unpubl.). Similar small effects of TSA addition were observed in studies of activation by SREBP-1a and Sp1 (Näär et al. 1998), suggesting that deacetylases may be limiting or may not play a role in this purified system. On the other hand, experiments studying the HIV-1 enhancer assembled in the presence of chromatin and HeLa nuclear extracts revealed a strong induction by TSA (Sheridan et al. 1997). To analyze the possible role of HDACs in our system, we included TSA in our transcription assays. Even at very high concentrations of TSA (0.5 μ M), there was no significant effect on pRB repression

by using either chromatin assembled with S190 extract or recombinant dNAP1/ACF (Fig. 5). Use of a second, potent HDAC inhibitor, trapoxin, yielded similarly negative results, and, furthermore, addition of purified, recombinant HDAC1 to our reconstituted system was also without effect (not shown). We verified that both HDAC inhibitors were active in an in vitro and in vivo setting (data not shown). These results are also consistent with our transient transfection studies in mammalian cells by using the same promoter. This suggests that whereas the recruitment of HDACs may be important in some circumstances for pRB repression, it is not absolutely required for inhibition of E2F activity or active repression of transcription from chromatin.

Discussion

The retinoblastoma protein has been shown to interact with several proteins, many of which play a role in tran-

Figure 3. Gal4-RB represses transcription on chromatin assembled with recombinant factors. (A) SDS-polyacrylamide gels of the purified factors used to assemble chromatin. The gels containing dNAP1 and ACF were Coomassie- and silver-stained, respectively. (B) Ethidium bromide-stained agarose gels of representative micrococcal nuclease assays to test the assembly of chromatin with S190 extract or recombinant ACF. Increasing amounts of micrococcal nuclease are indicated by the upward sloping triangle. (C) In vitro transcription using chromatin assembled with recombinant factors. Amounts of the indicated factors used were as in Fig. 2. Gal4-RB (12.5 ng) was used in lane 5.

scriptional and growth regulation. This observation implies that pRB may function through multiple mechanisms. The experiments presented here address this problem by using a simplified in vitro transcription assay able to support two distinct activities of pRB, direct inhibition of E2F and active repression. We have characterized previously the former activity, which can be explained most simply by the ability of pRB to inhibit the activation domain of E2F or to prevent or destabilize cooperative interactions between E2F and the PIC (Ross et al. 1999). Here, we have investigated active repression, which refers to the ability of pRB (in this case Gal4-RB) to repress the activity of proximally bound activator proteins (such as Sp1) to which pRB does not bind directly.

Remarkably, although Gal4-RB had no effect on proximal factors bound to a naked template, it potentially repressed transcription on a chromatin template. This observation allows us to unequivocally discriminate between two fundamentally distinct mechanisms of repression: one, active repression, requires chromatin whereas the other, direct inhibition, does not. The observation that Gal4-RB can efficiently repress transcription when incubated with a preassembled PIC further highlights the mechanistic difference between E2F inhibition and direct repression. In our previous studies of E2F inhibition on naked templates, a partially assembled PIC consisting of E2F, TFIIA, and TFIID was resistant to repression by pRB, suggesting that pRB functions in this setting by perturbing early events in the assembly of the preinitiation complex (Ross et al. 1999). These basal transcription factors may not be the

principal targets for active repression, and Gal4-RB may function by inhibiting an event after recruitment of the basal transcription machinery. pRB thus might inhibit recruitment of cofactors, or, alternatively, it might regulate events occurring later in (or after) initiation.

Taken together, the work presented here identifies an important role for chromatin in transcriptional regulation by pRB. Despite the requirement for chromatin in situations in which pRB represses transcription, our experiments suggest that, at least in some settings, neither direct inhibition of E2F nor active repression by pRB requires HDACs. These findings confirm and extend the notion that HDAC-mediated transcriptional repression is promoter specific in the context of fully assembled chromatin. Given the dispensability of HDACs for active repression by pRB here, our data indicate the possibility that pRB might inhibit transcription through a previously undefined target in chromatin. Possible targets could include chromatin remodeling factors or other proteins not associated with the basal transcription machinery. Future biochemical analyses should allow us to identify such potential chromatin-specific targets of pRB repression and

Figure 4. Prior assembly of a preinitiation complex (PIC) does not negate active repression of transcription by Gal4-RB. (A) Diagram of the order of addition experiments. In vitro transcription using (B) pG5Sp1E4-Gless and (C) p(E2F)4BGless templates assembled into chromatin with S190 extract. Sp1 (5 ng) and Gal4-RB (12.5 ng) were included in B, and 12.5 ng of E2F-4/DP-1 and 10 ng of Gal4-RB were added in C as indicated. Gal4-RB was added at the indicated times relative to PIC assembly. Relative transcription levels for B are listed under the lane numbers. Quantitation is relative to basal transcription (lane 1), which was set to unit value as described in the legend to Fig. 2.

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Figure 5. Addition of the HDAC inhibitor trichostatin A does not alleviate repression by Gal4-RB. In vitro transcription using chromatin assembled with S190 extract (lanes 1–6) or dNAP1 and recombinant ACF (lanes 7–12). Sp1 (5 ng), Gal4-RB (12.5 ng), and TSA (0.5 μ M) were included as indicated.

complexes with HDACs and SWI-SNF that might confer promoter-selective repression by pRB. Alternatively, pRB and its relatives could repress transcription in our system by recruitment of the corepressors RBP1 and CtBP (Lai et al. 1999; Meloni et al. 1999). These corepressors are thought to repress transcription in vivo without the need for HDAC recruitment, suggesting an additional histone deacetylase-independent mechanism for pRB repression.

Materials and methods

Cell culture, transfection, and CAT assays

C33A human cervical carcinoma cells were cultured in DMEM and transfected using standard calcium phosphate methods. Briefly, cells were transfected with 2 μ g of reporter plasmid, 3 μ g of pRSV- β gal, and indicated amounts of pCMV-Rb, pGal4-Rb, or pSG42Y (which encodes the Gal4 DNA-binding domain alone). The total amount of plasmid DNA was held constant at 15 μ g by supplementing with pBluescript (Stratagene). After a 12–16-h incubation, cells were washed with PBS and cultured for an additional 24 h in medium. Cells were harvested and processed for chloramphenicol acetyltransferase (CAT) and β -galactosidase assays with standard procedures by using [14 C]acetyl CoA (10 mCi/mmol) and liquid scintillation counting. Counts (cpm) were normalized according to β -galactosidase expression. Assays were performed in duplicate, and mean values are shown; representative data from three independent experiments are shown.

Baculovirus and plasmid construction

The Gal4-RB baculovirus was constructed by amplifying the Gal4-RB sequence from pM2-RB-FL (Weintraub et al. 1995), introducing *Pst*I and *Xma*I sites 5' and 3', respectively. The PCR product was digested with *Pst*I and *Sac*II, and the 500-bp product was isolated. An identical PCR product was digested with *Xma*I and *Sac*II, and the 2800-bp product was isolated. Both fragments then were ligated together into the *Pst*I/*Xma*I sites of pBacPAK8 (Clontech). The resulting plasmid, pGal4RB-BacPAK8, was cotransfected with BaculoGold DNA (PharMingen) into Sf9 cells according to the manufacturer's protocol to produce recombinant virus.

pG₅Sp1E4-Gless was constructed by inserting the *Xba*I-digested and T4 polymerase-blunted *Eco*RI fragment (141 bp encompassing the six Sp1-binding sites) from pS-G₅TL-141 (gift of F. Pugh, Pennsylvania State University) into the *Xba*I-digested and T4 polymerase-blunted *Bam*HI sites of pG₅E4Gless (gift of M. Carey, UCLA). p(E2F)₄BGless has been described previously (Ross et al. 1999). pG₅Sp1E4-CAT was prepared by inserting a *Hae*III promoter fragment from pG₅Sp1E4-Gless into a blunt-ended CAT reporter generated by excision of the cyclin E promoter from

pCyclin E-CAT (generous gift from J. Nevins, Duke University) with *Hind*III and *Xba*I.

Purification of recombinant proteins and general transcription factors
Gal4-RB was expressed by infecting High Five insect cells with recombinant Gal4-RB baculovirus. The protein was purified in two steps. First, extracts of infected cells were chromatographed on an affinity column containing a peptide from HPV E7 as described (Dynlacht et al. 1994). The eluate from this column was dialyzed and subsequently purified over a DNA affinity column bearing Gal4 binding sites essentially as described (Mizuguchi et al. 1997). Full-length pRB was expressed in insect cells and purified as described (Dynlacht et al. 1994). pRB expressed in insect cells has been shown to be predominantly hypophosphorylated (Dowdy et al. 1993). A fragment of Gal4 containing only the DNA-binding domain was expressed from the pGM1 plasmid and purified as described (Mizuguchi et al. 1997). Tubulin-tagged E2F-4 and GST-tagged DP-1 were purified and allowed to dimerize as described (Dynlacht et al. 1997). Alternatively, we also purified E2F-4/DP-1 complexes from insect cells coinfecting with HA-His₆-tagged E2F-4 and DP-1 viruses by using anti-HA antibody affinity chromatography. Flag-tagged Sp1 was expressed in High Five cells infected with a recombinant baculovirus (a gift from R. Tjian, University of California, Berkeley) and was purified using anti-Flag (M2) agarose (Sigma) affinity chromatography. Recombinant *Drosophila* His₆-tagged dNAP-1 was purified from baculovirus-infected High Five cells using Ni-NTA (Qiagen) affinity chromatography followed by FPLC MonoQ (Pharmacia) ion exchange chromatography. Recombinant ACF was purified from High Five cells coinfecting with baculoviruses encoding ACF-1 and Flag-tagged ISWI (Hamiche et al. 1999) by using anti-Flag (M2) agarose essentially as described (Ito et al. 1999). The basal transcription factors, including native TFIID, recombinant TFIIB, immunoprecipitated TFIID and RNA polymerase II, and a partially purified HeLa cell fraction containing TFIIE, TFIIIF, and TFIIH were prepared essentially as described (Ross et al. 1999). Histones and *Drosophila* embryo S190 extract were prepared as described (Näär et al. 1999).

In vitro transcription

In vitro transcription reactions were conducted using the G-less assay essentially as described (Ross et al. 1999), using the basal transcription factors indicated above. Purified ARC (Näär et al. 1999) was included in some of the transcription reactions, but because of significant levels of ARC in the partially purified TFIIE/F/H fraction, only modest increases in transcription were evident. Similar results always were obtained in the presence or absence of additional ARC. Transcription from chromatin templates was completely dependent on the addition of purified ARC when using a TFIIE/F/H fraction immunodepleted of ARC.

Transcription reactions were processed as in Ross et al. (1999), except that chromatin reactions reconstituted with S190 extract were treated with RNase T1 before gel electrophoresis. The transcription results presented are representative of experiments reproduced a minimum of three times. Relative transcription levels, where indicated, were determined by PhosphorImager analysis (Fuji).

Chromatin assembly and micrococcal nuclease assays

Chromatin assembly reactions were performed in 100- μ L reactions containing 0.5 μ g plasmid and 0.5 μ g core histones with either 25 μ L S190 extract or 4 μ g of dNAP1 and 50 ng of each subunit of ACF essentially as described (Ito et al. 1999), except that topoisomerase I was omitted. Micrococcal nuclease reactions were performed essentially as described (Bulger and Kadonaga 1994).

In vitro chromatin immunoprecipitation

Chromatin was assembled with S190 extract exactly as described above. After assembly, recombinant Sp1, Gal4-RB, or a combination of both was added as indicated, incubated at 30°C, and cross-linked with formaldehyde at a final concentration of 0.1% for 5 min at room temperature. Cross-linking reactions were terminated with glycine, and DNA was digested with micrococcal nuclease for 10 min at room temperature. Digestion was terminated with EDTA at a final concentration of 10 mM for 10 min at room temperature. Reactions were diluted with transcription buffer BC100 containing protease inhibitors (20 mM Hepes at pH 7.9, 0.1 mM EDTA, 20% glycerol, 0.1 M KCl, 1 mM AEBSE, 50 μ g/mL TLCK, 10 μ g/mL aprotinin, 1 μ g/mL leupeptin, 1 μ g/mL pepstatin) and incubated overnight (12 h) at 4°C with anti-Flag antibodies conjugated to

agarose (Sigma) to immunoprecipitate Flag-tagged Sp1. Immunoprecipitates were washed with BC100 containing 0.1% NP-40 and protease inhibitors. To reverse cross-links, we resuspended immunoprecipitates in SDS-PAGE sample buffer, and they were heated at 95°C for 1 h, electrophoresed, and immunoblotted with anti-pRB antibody G3-245 (Pharmingen). Blots then were stripped and reprobed with anti-Flag antibody (Sigma).

Acknowledgments

We are indebted to G. Schnitzler, R. Kingston, D. Fyodorov, and J. Kadonaga for helping us initiate chromatin reconstitution studies. We are especially grateful to R. Tjian and Y. Takahashi for comments on the manuscript and to R. Tjian for the Flag-Sp1 baculovirus and other critical reagents. We thank C. Wu for the Flag-ISWI baculovirus and D. Fyodorov and J. Kadonaga for the dNAP1 and ACF-1 baculoviruses. We are grateful to the National Cell Culture Center for providing us with HeLa cell pellets. We thank J. Lees for communicating unpublished data and D. Dean for generously providing plasmids. This work was supported by an NCI grant [CA77245-02] to B.D.D. B.D.D. also gratefully acknowledges the support of the Pew Scholars Program in the Biomedical Sciences.

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John F. Ross, Anders Näär, Hieu Cam, et al.

Genes Dev. 2001, **15**:

Access the most recent version at doi:[10.1101/gad.858501](https://doi.org/10.1101/gad.858501)

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