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S-Phase Progression Mediates Activation of a Silenced Gene in Synthetic Nuclei

ALISON J. CROWE, JULIE L. PIECHAN, LING SANG, AND MICHELLE C. BARTON*

Department of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, Ohio

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Aberrant expression of developmentally silenced genes, characteristic of tumor cells and regenerating tissue, is highly correlated with increased cell proliferation. By modeling this process in vitro in synthetic nuclei, we find that DNA replication leads to deregulation of established developmental expression patterns. Chromatin assembly in the presence of adult mouse liver nuclear extract mediates developmental stage-specific silencing of the tumor marker gene alpha-fetoprotein (AFP). Replication of silenced AFP chromatin in synthetic nuclei depletes sequence-specific transcription repressors, thereby disrupting developmentally regulated repression. Hepatoma-derived factors can target partial derepression of AFP, but full transcription activation requires DNA replication. Thus, unscheduled entry into S phase directly mediates activation of a developmentally silenced gene by (i) depleting developmental stage-specific transcription repressors and (ii) facilitating binding of transactivators.

Cellular commitment, differentiation, and specificity are determined primarily by the interaction of protein complexes with chromatin DNA. This gene-regulatory programming is challenged during each cell cycle by passage of the replisome during DNA synthesis. DNA replication may facilitate competition between transiently disrupted histones and transacting factors at the replication fork, potentially changing established chromatin structure and gene expression patterns (2, 7, 20, 29, 59). During tumorigenesis, terminally differentiated cells that have withdrawn from the cell cycle are induced to resume cycling, often through functional inactivation of tumor suppressor genes such as Rb and INK4a (30, 33). This cyclic disruption of chromatin structure provides a fertile environment, similar to that which exists during fetal development, for altering gene expression patterns. Aberrant adult activation of genes normally expressed only in the fetus is characteristic of many tumors (reviewed in references 31 and 39). For example, a hepatoma marker gene, alpha-fetoprotein (AFP), is transcriptionally repressed shortly after birth (reviewed in reference 52) and is reexpressed in mature hepatocytes only when they leave G_0 quiescence and begin cycling following partial hepatectomy or during hepatocellular carcinoma (HCC) (42; reviewed in reference 52). Expression of AFP is therefore closely linked to cell cycle progression; the renewal of DNA replication following transition from quiescence to S phase may play an epigenetic role in modulating gene expression.

The relative local concentration of transacting factors at the time of replication can determine whether a given chromatin structure is maintained or converted to an alternate conformation (17, 58). Modulating the balance of repressors and activators present during replication could be achieved through a variety of mechanisms, including functional inactivation of repressors and activation of transcription factors. Alternatively, the availability of transactivators could be controlled by shifting the replication timing of a given gene. Highly expressed genes, the majority of which replicate early in S phase, may bind limiting transcription factors, thereby depleting available pools for late-replicating silent or repressed genes (reviewed in reference 46).

To identify a potential role for DNA replication in renewed expression of a developmentally silenced gene, such as AFP, we have mimicked the reentry of differentiated G_0 cells into the cell cycle by synthetic nucleus assembly and S-phase progression in vitro using *Xenopus* egg extracts (25, 40, 41). We assessed the functional consequences of S-phase progression and DNA replication uncoupled from protein synthesis through in vitro transcription analysis of AFP expression. Using this approach, we have recapitulated the switch in AFP gene expression that occurs during HCC and liver regeneration from developmentally silenced to transcriptionally active. Our data provide the first direct evidence that deregulation of an established developmental expression pattern requires DNA replication. S-phase progression/DNA replication mediates activation of AFP in two ways: (i) by depleting a developmentally staged transcription repressor(s), thereby inhibiting the reestablishment of developmentally silenced templates postreplication, and (ii) by facilitating the binding of *trans*-acting factors to their sites in nucleosomal DNA.

MATERIALS AND METHODS

DNA templates. The AFP(3.8)-*lacZ* plasmid (51) contains 3.8 kb of sequence upstream of the AFP transcriptional start site, encompassing both enhancer I and the previously described developmental repressor domain (reviewed in reference 11). The 11-kb AFP(3.8)-*lacZ* plasmid was digested with *Eco*RI and *Cla*I enzymes to generate a 9.0-kb linear fragment encompassing the AFP enhancer I and promoter sequences fused to the *lacZ* gene. Biotin end-labeling reactions contained DNA (50 μg of DNA per 100-μl reaction volume), Klenow enzyme (NEB; 5 U per 100-μl reaction volume), 20 μM biotin 21-dUTP (Clontech, Palo Alto, Calif.), and dATP (20 μM) and were incubated for 30 min at 37°C to generate biotin-labeled *Eco*RI sites. Unincorporated deoxynucleotides, biotinylated deoxynucleotides, and small fragments of digested DNA are removed by gel filtration (Chromaspin 1000; Clontech).

Purified biotinylated DNA was coupled to streptavidin-coated M-280 Dynal paramagnetic beads in Kilobase Binding Buffer (Dynal Corporation, Oslo, Norway). Beads were washed twice in an equal volume of binding buffer. Biotinylated DNA (50 μg) was added to the resuspended beads to give a 1:1 final dilution. Coupling reaction mixtures were incubated at room temperature overnight on a rotating wheel. Coupled DNA-beads (solid-phase DNA templates) were washed three times with wash buffer (2 M NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA) using a magnetic concentrator (Dynal Corporation) and stored in wash buffer at 4°C (12, 13).

* Corresponding author. Mailing address: Dept. of Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH 45267-0524. Phone: (513) 558-5541. Fax: (513) 558-8474. E-mail: Michelle.Barton@UC.edu.

The 13.5-kb pAlbN albumin construct is a modification of pAN3-42 (generous gift of K. Zaret, Brown University). pAlbN contains 8.8 kb of albumin sequence including the albumin enhancer (−12.0 to −4.0 kb) fused at −800 bp of the albumin promoter relative to the transcription start site. The albumin sequences were linked to the *neo* coding region in a pUC18 backbone. Unique biotinylation and coupling restriction sites were generated by insertion of a polylinker (*Xma*I/*Asc*I/*Pac*I) at the *Sma*I site (+1837) of pAlbN. The DNA was digested with *Kpn*I and *Xma*I, biotinylated, and coupled to paramagnetic beads as described above.

PCR-mediated mutagenesis of AFP templates was conducted as previously described by Lee et al. (35) to generate templates deleted between −1000 and −541 or −209 of the AFP upstream regulatory region. Deletions between −1000 and −850, −765, or −586 were made using the Erase-A-Base system of mutagenesis, exactly as described by the manufacturer (Promega Corporation, Madison, Wis.). Endpoints of deletion and integrity of PCR-generated templates were determined by DNA sequencing.

Protein extracts and in vitro transcription. Adult mouse liver extract was prepared by the method of Gorski et al. (23). Hepatocarcinoma cell extracts were prepared from human HepG2 (ATCC catalog no. HB-8065) and mouse Hepa 1-6 (ATCC catalog no. CRL-1830) cells as described by Dignam et al. (14) with minor modifications (35). HeLa nuclear extract was prepared by the method of Workman and Abmayr (5). All extracts contained total proteins in concentrations of 5 to 10 mg/ml. *Xenopus* egg extracts were prepared as described previously (8). Fractionation by centrifugation of *Xenopus* egg clarified extract (LSS) yields a vesicular membrane fraction and a soluble high-speed supernatant (HSS) in *Xenopus laevis* (Xl) buffer (100 mM KCl, 4 mM $MgCl_2$, 10 mM potassium HEPES [pH 7.2], 100 mM sucrose, 0.1 mM EGTA). Generally, chromatin was assembled in membrane-depleted HSS prior to synthetic nucleus formation in crude, clarified LSS. Where noted, HSS plus membrane vesicles have been used in transcription and replication assays as described previously (7). Replication assays were also performed in LSS as described below.

In vitro chromatin assembly and transcription reactions were performed as described previously (12). Cellular extracts were supplemented with nuclear dialysis buffer (NDB: 20 mM HEPES [pH 7.9], 50 mM KCl, 0.5 mM EDTA, 20% glycerol, 2 mM dithiothreitol [DTT]) to an equal volume in all reactions (a total of 25 μl). These were added to 500 ng of solid-phase DNA templates during a 20-min room temperature preincubation or as indicated by the experimental design (final protein concentration during preincubation, 8 μg/μl). Following preincubation, HSS supplemented with ATP to 6 mM, poly(dI:dC) to 4 ng/μl, and 1 M $MgCl_2$ to a 10 mM final concentration was added to give a total of 50 μl (usually 600 to 800 μg of protein) and incubated for 1 h at 22°C. In order to inhibit any background DNA replication during the chromatin assembly period, the DNA polymerase inhibitor aphidicolin was added to each reaction at a 40-ng/μl final concentration. Chromatin-assembled solid-phase DNA was washed three times by placement on a magnetic concentrator (magnetic concentration) and removal of unbound extract and proteins, followed by addition of 200 μl of NDB (containing 0.01% NP-40) to each reaction. Chromatin can be transcribed immediately or assembled into synthetic nuclei prior to in vitro transcription. Washed chromatin templates were assembled into synthetic nuclei by addition of approximately 1.5 mg of total protein of LSS (supplemented with ATP and $MgCl_2$ to give final concentrations in the assembly reaction mixture of 3 mM ATP and 5 mM $MgCl_2$), plus cellular extracts or NDB in a total volume of 50 μl. Reaction mixtures were incubated for 2 h at 22°C. Templates were washed as described above prior to in vitro transcription under standard transcription conditions (12).

Synthetic nucleus analysis. Nuclear transport studies were performed by the method of Gorlich et al. (22). Briefly, 1 μg of AFP(3.8)-*lacZ* solid-phase bead-DNA was incubated in 100 μl of unfractionated *Xenopus* egg extract (LSS) containing 1 mM ATP, 0.2 mM GTP, 10 mM creatine phosphate (Boehringer Mannheim), and 9.5 U of creatine kinase (Boehringer Mannheim) per ml to form solid-phase synthetic nuclei. After 1.5 h, 150 ng of the indicated recombinant fusion protein (LEF-1:eGFP or GST:eGFP) stored in 25 mM Tris (pH 8.0)–50 mM ammonium sulfate–5% glycerol–1 mM EDTA–1 mM DTT was added and further incubated in transport buffer (final concentrations: 4 mM HEPES [pH 7.8], 22 mM potassium acetate, 1.0 mM sodium acetate, 0.4 mM magnesium acetate, 0.2 mM EGTA, 0.4 mM DTT). After 30 min, 2-μl aliquots were removed and mixed with 2 μl of the DNA-specific stain propidium idodide (Oncor, Washington, D.C.). Nuclei were analyzed by fluorescent and phase-contrast microscopy at a magnification of ×400.

DNA replication was assayed by steady-state "continuous labeling" of newly synthesized DNA with 1 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; ICN) present during the entire incubation period with *Xenopus* egg extract (50). DNA replication reactions were performed with 500 ng of bead-DNA in (as noted in the figures or text) 25 μl of LSS alone, 25 μl of HSS plus membrane vesicles (7), or sequential HSS incubation, washes, and LSS incubation conditions described for in vitro transcription (see above), plus NDB or cellular extracts (100 to 120 μg) in a total volume of 50 μl. Replication assays were terminated by addition of 2.5% sodium dodecyl sulfate–60 mM EDTA. DNA was purified by overnight digestion with proteinase K (0.8 mg/ml) at 37°C followed by phenol-chloroform (1:1) and chloroform extractions and ethanol precipitation prior to gel electrophoresis on a Tris–borate–EDTA–0.8% agarose gel. The proteinase digestion step also severs the biotin-streptavidin linkage between the biotinylated template and the paramagnetic bead support. Replicated DNA was visualized by autoradiography of the dried gel.

Relative levels of full-length DNA replication within solid-phase nuclei were quantified by ImageQuant analysis of scanned autoradiograms comparing full-length replicated DNA products under each of the incubation conditions noted. Nucleosome assembly of the ^{32}P-labeled, replicated AFP-bead DNA was assessed by micrococcal nuclease (MNase; Boehringer Mannheim) digestion after a 2-h incubation with LSS containing 3 mM ATP, 5 mM $MgCl_2$ and 1 μCi of [α-^{32}P]dATP (3,000 Ci/mmol; ICN). Samples were digested and analyzed as described previously (8). MNase protection patterns were identical whether samples were incubated in LSS or HSS plus membrane vesicles (8).

RESULTS

Hepatoma-specific derepression of chromatin-assembled AFP templates. The tumor marker gene AFP provides an ideal model for studying aberrant gene activation due to its strict tissue-specific and developmental stage-specific regulation in vivo. AFP is highly expressed during fetal development in endoderm-derived tissues, including the yolk sac, gut, and liver. Developmental stage-specific silencing occurs shortly after birth, and under normal circumstances, AFP expression remains repressed in the differentiated hepatocyte throughout adult life (reviewed in references 11 and 52). However, if hepatocytes are induced to resume cycling, as occurs both during HCC and after liver damage, the silenced AFP gene is reactivated. This is in contrast to the evolutionarily related albumin gene, which lies directly upstream of AFP. Albumin levels remain relatively constant throughout adult life, and these high levels are maintained during both liver regeneration and HCC (36).

To identify the molecular mechanisms responsible for the aberrant reactivation of AFP, we have established in vitro chromatin and synthetic nucleus transcription systems for the AFP gene. Based on a design for transcriptional analysis of the *Drosophila* hsp70 promoter (47, 55), we have coupled the AFP template to streptavidin-coated paramagnetic beads. The generation of immobilized templates facilitates washing of chromatin-assembled templates and removal of any unbound proteins present in cellular or *Xenopus* assembly extracts prior to in vitro transcription. The details of biotin end labeling, DNA coupling, and manipulation of the bead-DNA (solid phase) templates in *Xenopus* chromatin and synthetic nucleus assembly systems have been described elsewhere (12, 13). As diagrammed in Fig. 1A, solid-phase AFP templates containing 3.8 kb of upstream AFP regulatory sequence including enhancer I and the distal and proximal promoter elements are assembled into chromatin by incubation with fractionated *Xenopus* egg extract (HSS) (41, 45).

To program the AFP gene in either its developmentally silenced or tumorigenically active state, we have incubated the gene with tissue or cellular extracts prior to chromatin assembly. We have used the AFP- and albumin-expressing HCC cell lines human HepG2 and mouse Hepa 1-6 as sources of hepatoma *trans*-acting factors. Adult mouse liver (ML) extract was used to provide proteins present in a differentiated tissue where AFP is developmentally silenced and albumin is constitutively transcribed. As shown in Fig. 1B, preincubation of the AFP gene with HepG2 extract established a transcriptionally active AFP chromatin template (lane 3), resulting in a 27-fold increase in expression over a chromatin-repressed template (lane 1). Preincubation with ML extract (lane 2) resulted in only very low levels of AFP transcription (1.8-fold over a chromatin-repressed template). This developmentally silenced state of AFP, established by chromatin assembly in adult ML extract, is resistant to magnetic concentration, isoosmotic washing, and transcription. In contrast, both ML extract and

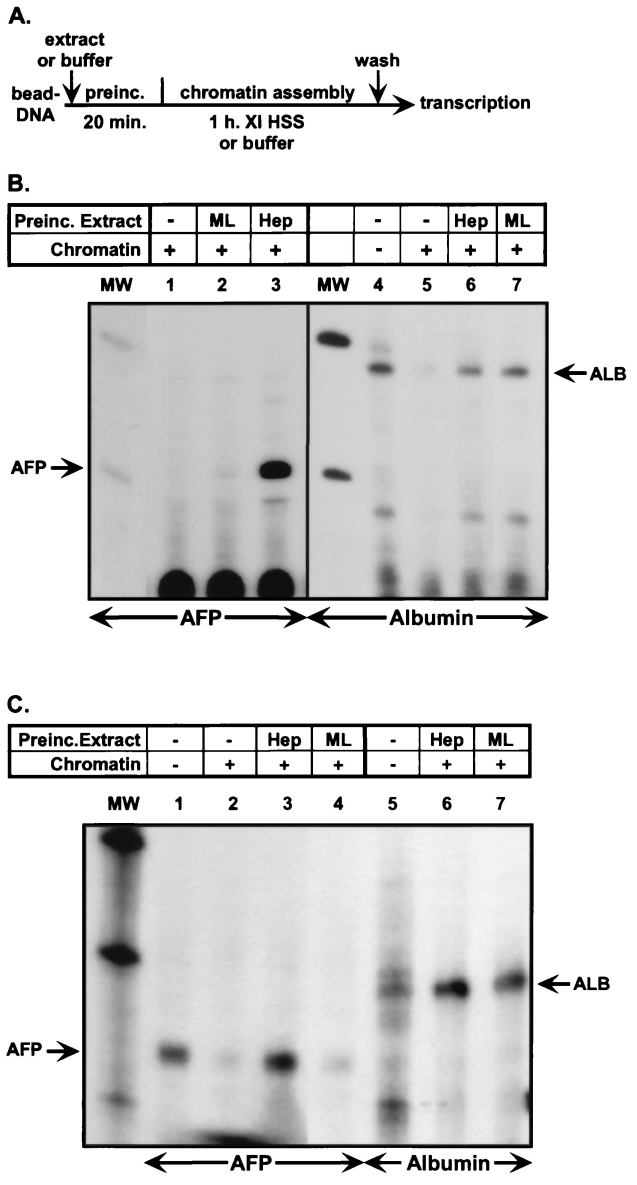

FIG. 1. Reconstitution of in vivo expression patterns in vitro. (A) Diagram of coupled chromatin assembly-transcription system. (B) Hepatoma-specific derepression of AFP and albumin chromatin templates. Immobilized AFP (lanes 1 to 3) or albumin (lanes 4 to 7) templates were preincubated (preinc.) in NDB (lanes 1 and 5), adult mouse liver (ML) extract (lanes 2 and 7), HepG2 (lane 3), or Hepa 1-6 extract (lane 6) prior to chromatin assembly in HSS. Chromatin templates were washed and in vitro transcribed in HeLa extract. A nucleosome-free albumin template (lane 4) was in vitro transcribed in HeLa extract (lane 4). (C) Differential regulation of AFP and albumin in simultaneous transcription reactions. Immobilized AFP and albumin templates were coincubated with either NDB (lanes 1, 2, and 5), HepG2 (lanes 3 and 6), or ML (lanes 4 and 7) extract prior to chromatin assembly in HSS (lanes 2 to 4 and 6 to 7) or incubation in Xl buffer in the absence of chromatin assembly (lanes 1 and 5). All templates were washed and in vitro transcribed in HeLa extract. After RNA isolation, each reaction mixture was divided in two, and primer extension analysis was performed with either an AFP-specific primer (lanes 1 to 4) or an albumin-specific primer (lanes 5 to 7). The extension products for AFP (84 bp) and albumin (ALB; 105 bp) are indicated. Radiolabeled ϕX174 DNA digested with *Hae*III was run as a molecular size standard (lane MW).

Hepa 1-6 extract established transcriptionally active albumin chromatin (lanes 6 and 7).

Similar results were obtained when AFP and albumin templates were present in the same chromatin assembly and transcription reaction mixtures and then divided in half for separate primer extension analysis (Fig. 1C). Differential expression of repressed AFP (lane 4) and activated albumin (lane 7) by chromatin assembly in ML extract recapitulated regulated expression in differentiated hepatic cells. Preincubation with HepG2 extract established transcriptionally active albumin (lane 6) and AFP (lane 3) templates. HepG2 extract was used as a source of hepatoma factors in all of the remaining experiments. The only observed difference in expression of AFP and albumin templates in vitro between mixed templates and those transcribed in parallel is an apparent loss of albumin transcription start site integrity (lane 5). Whether the two mixed templates in naked DNA transcriptions compete for factors that maintain the start site of transcription is unknown at this time.

Together, these data indicate that solid-phase chromatin transcription accurately reflects the expression patterns observed in HCC and differentiated liver. Thus, the ML extract contains all the necessary transactivators to activate a chromatin-assembled gene, such as albumin, that is normally expressed in adult liver. There is likely a balance of transactivators and repressors present in the adult liver that directly bind both AFP and albumin genes to generate the silenced and active states, respectively.

Solid-phase synthetic nuclei support nuclear protein transport and DNA replication. In vivo activation of the AFP gene in adults occurs only under conditions of rapid cellular proliferation such as exist during liver regeneration and HCC. We reasoned that derepression of AFP after chromatin assembly in vitro may require on-going DNA replication. To test this hypothesis directly, we needed to assemble functionally competent synthetic nuclei with AFP DNA coupled to streptavidin beads. Addition of the vesicular fraction of *Xenopus* egg extract or unfractionated *Xenopus* egg cytoplasm (LSS) to chromatin DNA results in the formation of a nucleus-like structure around cloned DNA, which enables nuclear functions of protein transport and semiconservative DNA replication (41).

Nuclei that form around DNA-coupled 2.4-μm paramagnetic beads (solid-phase nuclei) incubated in interphase LSS (41) are competent for both nuclear transport and DNA replication (Fig. 2) (27). As shown in Fig. 2A, solid-phase nuclei transport proteins in a nuclear localization signal (NLS)-dependent manner. Recombinant transcription factor LEF-1 protein, which contains a highly basic B-box NLS, fused to an enhanced green fluorescent protein (eGFP) tag (a generous gift from M. Prieve and M. Waterman) is efficiently localized by nuclear transport within preformed solid-phase nuclei (Fig. 2A, panel b). Transported LEF-eGFP appears as a green, fluorescent ring (b) around the DNA-coupled paramagnetic beads (c) encapsulated by a membrane (a). No concentrated green fluorescence is detected when LEF-eGFP is added to DNA-coupled beads in the absence of nucleus formation (d), or when a glutathione *S*-transferase (GST)–eGFP fusion protein lacking an NLS is added to the preformed nuclei (Fig. 2A, panel e), demonstrating the specificity of the observed transport. The yellow fluorescence observed after long exposure times in these latter two cases is due to intrinsic fluorescence of the beads themselves. These data show that solid-phase nuclei selectively transport proteins in an NLS-dependent manner, confirming the functional integrity of the nuclear membrane.

DNA replication within solid-phase nuclei formed by incubation in *Xenopus* egg extract LSS was measured by steady-state levels of [α-^{32}P]dATP-labeled DNA accumulated over time (Fig. 2B). Radioactive incorporation is specifically blocked by addition of a DNA polymerase inhibitor, aphidicolin, indicating that radiolabel incorporation is polymerase specific (Fig. 2B, lane 5). Slower-migrating, unresolved replication

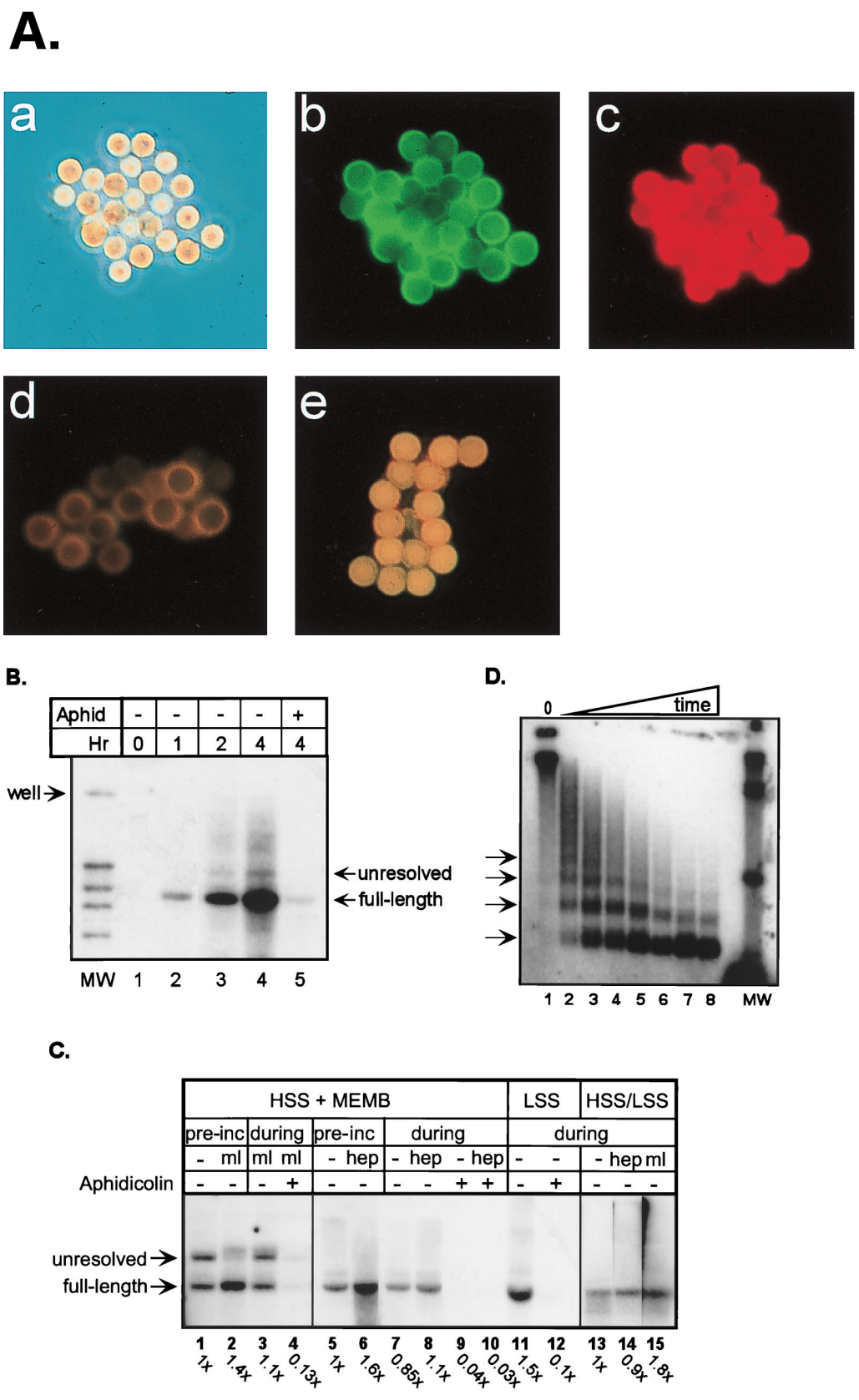

FIG. 2. Solid-phase synthetic nuclei. (A) Solid-phase synthetic nuclei support nuclear transport. Immobilized AFP templates were assembled for 1.5 h in LSS to generate solid-phase synthetic nuclei (a to c and e), or in Xl buffer alone (d). Assembly reaction mixtures were further incubated for 30 min with approximately 150 ng of either LEF-1:eGFP (a to d) or GST:eGFP (e). After addition of propidium iodide, samples were visualized by phase-contrast microscopy (a) or by fluorescence microscopy (b to e) with either a GFP wide-band filter (b, d, and e) or a rhodamine filter (c). In the absence of a nuclear membrane (d) or an NLS (e), no concentrated

intermediates or intertwined daughter molecules are often apparent (Fig. 2B and C). We have previously confirmed that DNA radiolabeled under steady-state conditions is the end product of semiconservative DNA replication and not gap repair by methylation state analysis with the isoschizomers *Dpn*I and *Sau*3AI (12). Relative replication levels were assessed under a number of incubation conditions that were later used in transcription analyses (Fig. 2C): preincubation in buffer only, adult ML, or HepG2 hepatoma cell extract, followed by assembly in HSS plus membrane vesicles, LSS only, or sequential HSS, washes, and LSS incubation, as indicated. Under each incubation condition, a baseline was established by addition of buffer only, followed by nucleus assembly and replication in the presence of [α-^{32}P]dATP, DNA purification, and separation by gel electrophoresis. Comparison of replicated, full-length DNA reveals a slight stimulation of replication in ML extract of 1.4-fold when preincubated (Fig. 2C, lane 2) but not when added during nucleus assembly (lane 3) in HSS plus membrane vesicles. Similarly, HepG2 extract enhanced replication when preincubated with the template (1.6-fold; Fig. 2C, lane 6), but not when added during assembly (lane 8). Aphidicolin addition to HSS and membrane vesicles inhibited replication by 87 and 97% in the presence of ML extract and hepatoma extract, respectively (Fig. 2C, lanes 4 and 10), and by 93% when added to LSS assembly reactions (lane 12). Addition of ML extract to preassembled developmentally repressed chromatin templates during nucleus assembly in LSS (Fig. 2C, lane 15) again had a stimulatory effect (1.8-fold) on DNA replication. These results indicate that ML factors may slightly increase the efficiency of DNA replication in synthetic nuclei. No increase was detected when hepatoma extract was added during nucleus assembly of preassembled chromatin templates (Fig. 2C, lane 14).

To assess the chromatin structure of newly replicated DNA, solid-phase AFP templates assembled into synthetic nuclei in LSS were replicated in the presence of [α-^{32}P]dATP followed by digestion with MNase (Fig. 2D). A time course of digestion with MNase revealed extensive assembly of nucleosomes on the newly replicated DNA. Nucleosomal protection of DNA occurred with a spacing of approximately 160 to 180 bp, identical to that observed with uncoupled DNA assembled into chromatin by *Xenopus* egg extract (data not shown) (9).

DNA replication mediates hepatoma-specific activation of chromatin-repressed AFP. After establishing that solid-phase synthetic nuclei supported both nuclear transport and DNA replication, we directly tested whether DNA replication was required for hepatoma-induced reprogramming of AFP gene expression. To recreate the proliferative environment present in hepatoma cells, AFP templates were assembled into synthetic nuclei by incubation in fractionated *Xenopus* egg extract (HSS) plus membrane vesicles. These conditions have been shown previously to direct stepwise chromatin assembly and synthetic nucleus formation (41, 49), capable of derepressing the β-globin promoter in the presence of transactivating red blood cell extracts (7).

We assayed the ability of hepatoma factors to activate chromatin-repressed AFP templates under replicating and nonreplicating conditions, as illustrated in Fig. 3A. Complete derepression of nucleosome-assembled templates occurred only when hepatoma extract was provided either prior to chromatin assembly/replication (Fig. 3B, lane 2) or during DNA replication (1 to 1.5 h after *Xenopus* extract addition; lane 5). As the majority of nuclei are already assembled after a 1-h incubation in HSS plus membranes (41), proteins added after this point (Fig. 3B, lanes 4 to 9) must likely be actively transported across the nuclear membrane in order to influence gene regulation. However, even under these stringent conditions, transcription levels measured on chromatin-repressed templates (Fig. 3B, lane 1) replicated in the presence of hepatoma extract (10.2-fold derepression; lane 5) were 91% of those obtained by hepatoma preincubation (11.3-fold derepression; lane 2), demonstrating a nearly complete reversal of the chromatin-repressed state during replication.

Chromatin-mediated repression of AFP gene expression was maintained during replication in the absence of cellular extract (Fig. 3B, lane 4), indicating that the physical process of DNA synthesis alone is insufficient to activate AFP transcription. Addition of ML extract during DNA replication (Fig. 3B, lane 6) resulted in maintenance of the repressed state; it is unclear whether this repression is propagated through passive nucleosome assembly, as in lane 4, or reflects the AFP-silenced, differentiated hepatic state imposed by ML extract (lane 3), or both. These two models of ML-mediated repression during DNA replication have been further dissected, as described below.

Hepatoma-specific proteins activated a low level of transcription (25%, comparing lane 8 with lane 5 in Fig. 3B) when added to solid-phase nuclei in the presence of a replication inhibitor (aphidicolin). As we have seen that aphidicolin inhibits DNA replication 97% under these conditions (Fig. 2C, lane 10), the hepatoma-specific activation observed in lane 8 is most likely not mediated by residual DNA replication. These data suggest either a limited ability of hepatoma factors to bind their sites in nucleosomal DNA or the presence of chromatin-remodeling activity, or both. ML extract was unable to derepress chromatin during this postassembly incubation (Fig. 3B, lane 9). Importantly, chromatin remodeling factors present in the *Xenopus* egg extract itself are insufficient to activate a chromatin-repressed AFP gene (Fig. 3B, lane 7).

DNA replication mediates activation of developmentally silenced AFP in vitro. These results suggest that DNA replication facilitates binding of hepatoma activators to a chromatin-repressed template, resulting in derepression and transactivation

GFP fluorescence was observed. (B) Steady-state DNA replication. Immobilized AFP templates were assembled into synthetic nuclei in the presence of [α-^{32}P]dATP and incubated for 0 (lane 1), 1 (lane 2), 2 (lane 3), or 4 (lane 4 and 5) hours in LSS. Aphidicolin (40 ng/μl) was added to lane 5. Purified DNA was subjected to electrophoresis and visualized by autoradiography of the dried gel. Labeled arrows point to replicated full-length DNA, unresolved replication intermediates or concatemers, and the location of the well. Radiolabeled lambda DNA digested with *Hin*dIII was run as molecular size standards (lane MW). (C) DNA replication under transcription conditions. Immobilized AFP templates were assembled into synthetic nuclei by preincubation (preinc.) in HSS plus membranes (MEMB) (lanes 1 to 10) or in LSS (lanes 11 and 12) or by preassembly in HSS followed by nucleus assembly in LSS (lanes 13 to 15). Templates were incubated with either NDB (lanes —) or adult ML (ml), or HepG2 (hep) extract at the indicated times during assembly. Templates in lanes 13 to 15 were preincubated with ML extract prior to assembly in HSS, and these silenced templates were then assembled into nuclei in the presence of the indicated extracts. Incubations and assembly reactions were performed exactly as diagrammed in Fig. 3 and 4. DNA replication was monitored by inclusion of [α-^{32}P]dATP during synthetic nucleus assembly. Purified DNA was subjected to electrophoresis and visualized by autoradiography of the dried gel. Labeled arrows point to replicated full-length DNA and unresolved replication intermediates. Relative replication levels (fold) in comparison to buffer controls are indicated below the lanes. (D) Nucleosome assembly on replicated DNA. Immobilized templates were assembled into synthetic nuclei in LSS in the presence of [α-^{32}P]dATP for 2 h. DNA was then digested with MNase, and aliquots were withdrawn for analysis at 0, 2.5, 5, 10, 20, 40, 60, and 80 min. Nucleosome spacing was estimated by comparison to a 123-bp DNA ladder (Gibco-BRL) and radiolabeled λ *Hin*dIII fragments (lane MW). Arrows point to MNase-protected fragments.

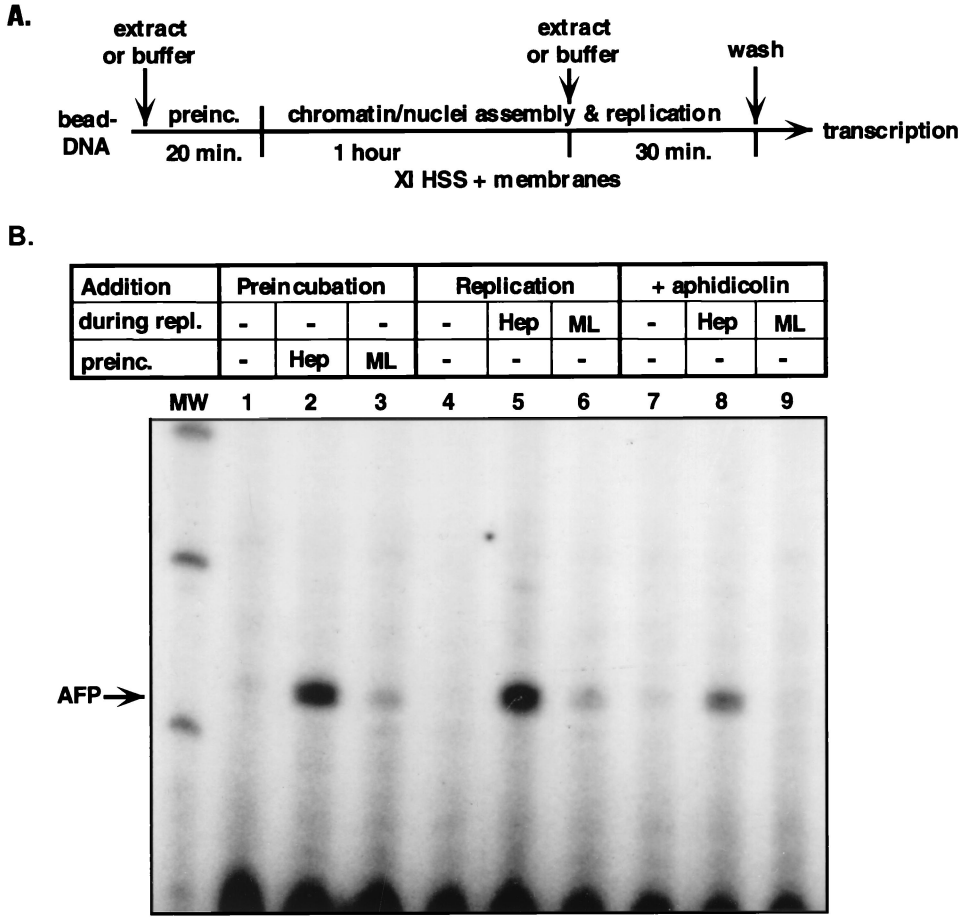

FIG. 3. Hepatoma-specific activation of AFP during DNA replication. (A) Diagram of solid-phase nucleus assembly and transcription system. (B) In vitro transcription. Immobilized AFP templates were incubated with NDB (lanes 1 and 4 to 9) or HepG2 (lane 2) or ML (lane 3) extract. Templates were assembled into synthetic nuclei by incubation in HSS plus membranes. Synthetic nuclei were further incubated for an additional 30 min in the presence of NDB (lanes 1 to 4 and 7) or HepG2 (lanes 5 and 8) or ML (lanes 6 and 9) extract. Aphidicolin (lanes 7 to 9) was added concomitantly with the HSS plus membranes. All samples were washed and in vitro transcribed in HeLa extract. Radiolabeled ϕX174 DNA digested with *Hae*III was run as molecular size standards (lane MW).

of the chromatin template. In the above experiments, chromatin-repressed AFP templates were used as the starting material to examine the effects of DNA replication on AFP gene expression. However, in the adult liver, repression of AFP occurs through an active process involving developmental stage-specific repressor proteins (35, 54). To more accurately model the developmentally silenced state of AFP, we employed a two-stage chromatin-synthetic nucleus assembly system (38) coupled to in vitro transcription (Fig. 4A). In the first stage, developmentally silenced AFP templates were generated by preincubation with ML extract and chromatin assembly in HSS, followed by an isoosmotic wash step to remove all protein not interacting with DNA or chromatin. Silenced templates were then exposed to one of two pathways in the second stage: (i) stable maintenance of chromatin templates by incubation in buffer only, or (ii) formation of synthetic nuclei and semiconservative replication of chromatin-assembled templates by incubation in unfractionated *Xenopus* egg extract (LSS). Using this approach (sequential HSS-LSS incubations), the functional consequences of synchronized DNA replication (38) within solid-phase nuclei (pathway 2) can be assessed independently of nuclear transport and under conditions of limited or excess protein or extract concentration.

After passage of a replication fork, *trans*-acting factors must bind their respective sites on the newly replicated DNA in order to reestablish a given gene expression pattern. The local concentration of factors during chromatin reassembly would therefore be expected to influence the outcome of this process. To determine whether the concentration of ML proteins present during replication might affect the reassembly of developmentally silenced templates postreplication, we replicated the silenced templates in buffer only. As shown in Fig. 4B, we found that, unlike our results with chromatin-repressed AFP templates, DNA replication of developmentally silenced AFP chromatin templates in the absence of any additional cellular extracts resulted in transcription activation (5.3-fold; Fig. 4B, lane 2). Limiting the concentration of adult ML extract, through washing away of unbound proteins, activated AFP transcription in a DNA replication-dependent manner (Fig. 4B, compare lanes 1 and 2). Therefore, DNA replication may mediate the loss of sufficient DNA-bound transcription repressors to inhibit reformation of silencing complexes on the replicated DNA.

Addition of hepatoma extract during DNA replication of developmentally silenced templates increased transcription only 1.2- to 2-fold compared with the effect of replication alone

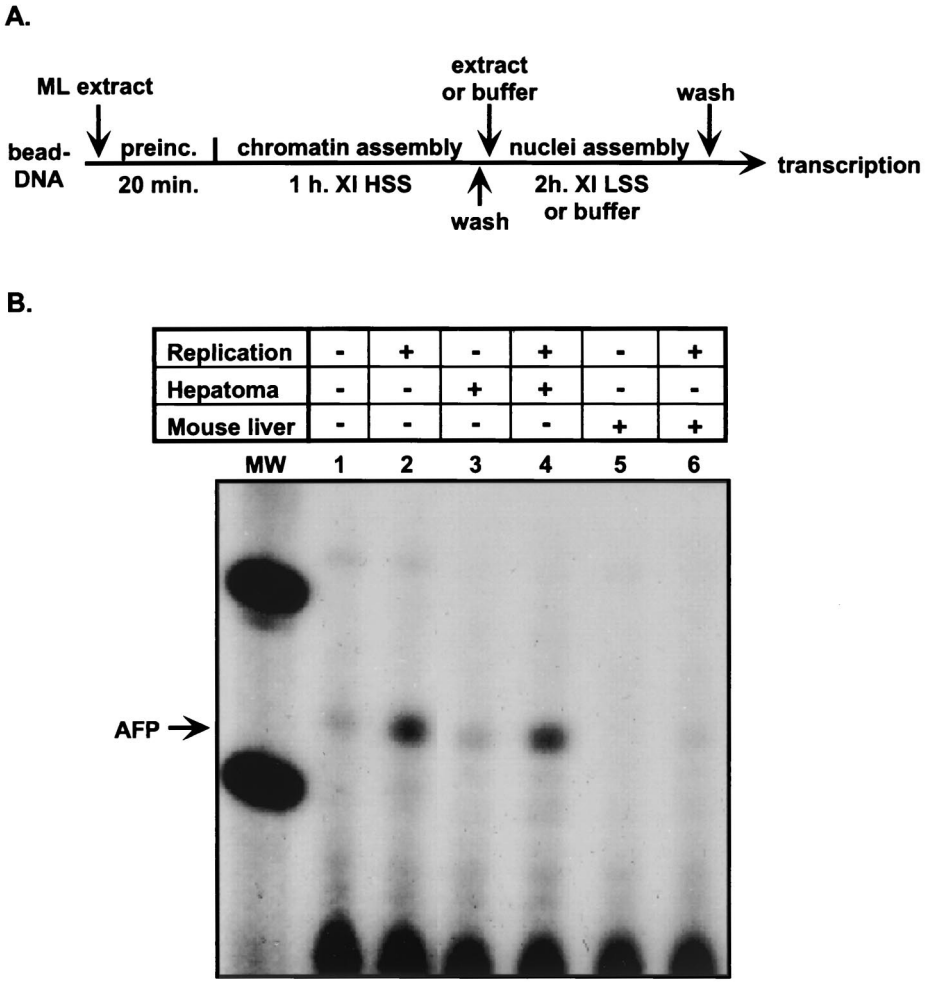

FIG. 4. DNA replication alone is sufficient to activate a developmentally silenced AFP template. (A) Diagram of two-step solid-phase nucleus replication and transcription system. (B) In vitro transcription. Immobilized AFP templates were incubated with ML extract (lanes 1 to 6) prior to chromatin assembly. Chromatin templates were washed and either incubated in Xl buffer (lanes 1, 3, and 5) or further assembled into synthetic nuclei by incubation with LSS (lanes 2, 4, and 6). NDB (lanes 1 and 2), HepG2 extract (lanes 3 and 4), or ML extract (lanes 5 and 6) was included during the 2-h postchromatin/nucleus assembly period. All samples were washed and in vitro transcribed in HeLa extract. Radiolabeled ϕX174 DNA digested with *Hae*III was run as molecular size standards (lane MW).

(Fig. 4B, compare lanes 4 and 2). However, as shown in Fig. 3B, both HepG2 extract and DNA replication are required to activate a chromatin-repressed template. These data suggest that further supplementation with hepatoma factors does not significantly amplify replication-mediated activation of developmentally silenced AFP. Additionally, under these "washed chromatin" conditions, HepG2 extract added in the absence of replication (Fig. 4B, lane 3) was unable to alter the repressed state of AFP, whereas replication-independent activation by HepG2 was observed on the "unwashed" chromatin templates (Fig. 3B, lane 8). Chromatin remodeling complexes present in the *Xenopus* egg extract may therefore participate in the replication-independent activation.

Depletion of developmental repressor(s) plays a role in AFP activation. Replication-mediated depletion of key repressors should occur only under limiting protein conditions. The paramagnetic bead concentration and isoosmotic washes of chromatin performed prior to solid-phase nucleus formation imposed these conditions (Fig. 4A). Based on this assumption, replication of the silenced template in the presence of excess ML extract should negate the repressor titration effect as efficiently as inhibition of DNA replication does. As shown in Fig. 4B, addition of ML extract during replication resulted in maintenance of the silenced state (lane 6). Analysis of the DNA replication levels under these conditions (Fig. 2C, lane 15) indicated that the presence of ML extract during replication led to a 1.8-fold increase in replication. Thus, even under enhanced replication conditions, excess ML extract was sufficient to maintain the silenced state.

The activation observed upon replication of the ML-silenced AFP template suggests that duplication of the target DNA may result in titration of critical, developmental stage-specific repressors, allowing ML activators to reassemble a transcriptionally competent template. We reasoned that if increasing the DNA concentration by DNA replication is sufficient to deplete a repressor(s) under limiting adult ML extract conditions, then exogenous addition of increasing amounts of AFP DNA during replication in excess adult ML extract should mimic this effect and activate transcription. To test this possibility directly, we replicated ML-silenced AFP chromatin in excess ML extract plus various concentrations of supercoiled AFP DNA (Fig. 5). Addition of AFP DNA blocked excess adult ML-mediated repression (Fig. 5B, compare lanes 3 and 4 to lane 2). Derepression of AFP transcription was also detected following ad-

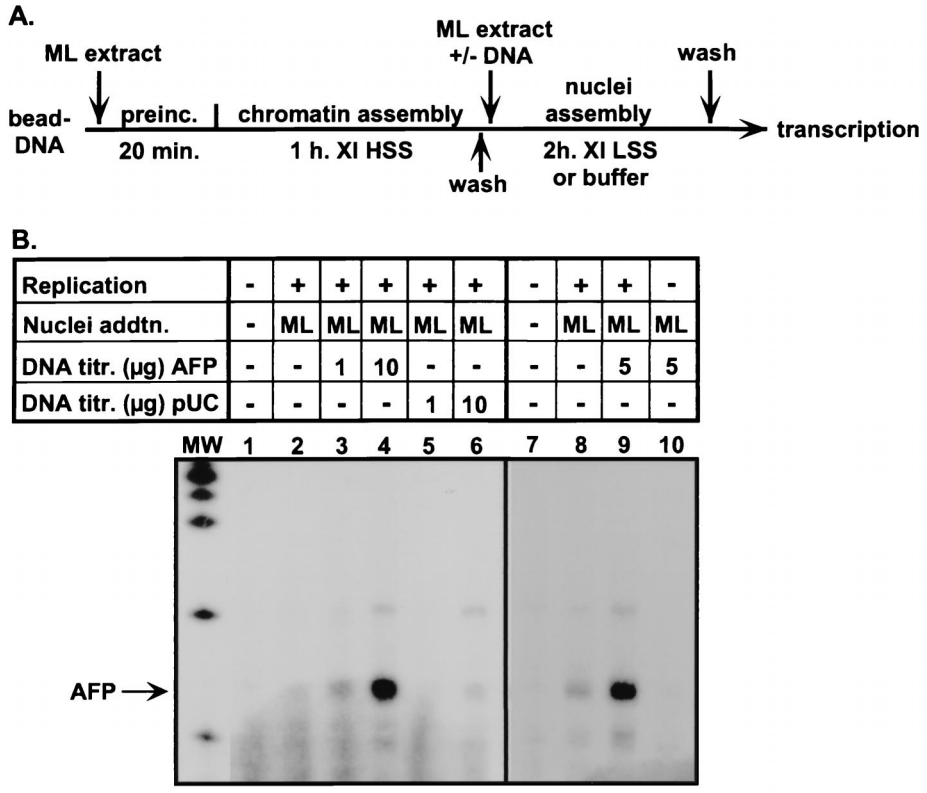

FIG. 5. Sequence specificity and replication dependence of repressor depletion. (A) Diagram of two-step solid-phase nucleus replication and transcription system. (B) In vitro transcription. Immobilized AFP templates were incubated with ML extract prior to chromatin assembly in HSS (lanes 1 to 10). Chromatin templates were washed and further incubated in the presence (lanes 2 to 6 and 8 to 10) or absence (lanes 1 and 7) of LSS to assemble synthetic nuclei. Aphidicolin was added during synthetic nucleus formation to block DNA replication in lane 10. ML extract was added during synthetic nucleus formation (lanes 2 to 6 and 8 to 10). The indicated amount of supercoiled AFP(3.8)-*lacZ* DNA (lanes 3, 4, 9, and 10) or supercoiled pUC DNA (lanes 5 and 6) was added as a competitor during synthetic nucleus formation. All samples were washed and in vitro transcribed in nuclear HeLa extract. Radiolabeled ϕX174 DNA digested with *Hae*III was run as molecular size standards (lane MW).

dition of competitor AFP DNA that lacked the *lacZ* coding region (data not shown). Titration of repressors by AFP DNA required the physical process of replication, as inclusion of aphidicolin maintained the repressed state (Fig. 5B, compare lanes 9 and 10). Together, these results strongly support our hypothesis that DNA replication mediates tumor marker gene activation both by facilitating binding of transactivators and through depletion of repressor proteins.

To determine whether depletion of a repressor(s) was due to competition in *trans* by sequence-specific binding, we compared the ability of nonspecific pUC DNA versus AFP DNA to compete for repressor binding. Addition of equivalent amounts of pUC DNA (Fig. 5B, lanes 5 and 6) had no effect on AFP expression, indicating that depletion of a repressor(s) requires the presence of AFP-specific sequences. Importantly, the sequence specificity of the activation rules out the formal possibility that excess DNA nonspecifically depleted histones from the LSS, reducing the capacity for chromatin-mediated repression. Studies of factors bound to competitor DNA and the chromatin structure of the competitors after replication-mediated depletion are ongoing. These data indicate that sequence-specific repressors can be depleted from the local environment during DNA replication, thereby inhibiting the reformation of a transcriptionally silenced template.

Repression of AFP transcription is sequence specific. To begin characterization of the developmental repressors depleted by DNA replication, we transcribed AFP templates deleted within the developmental repressor region, a broadly defined 750-bp region (−1000 to −250) that mediates postnatal repression of AFP expression in transgenic analysis (54). AFP DNA was deleted by PCR-mediated mutagenesis from −1000 to −541 (removing approximately one-half of the developmental repressor region) and from −1000 to −209 (removing the entire developmental repressor region). RNA transcribed from deletion templates is distinguished from full-length primer extension products by PCR-engineered removal of a 13-nucleotide polylinker connecting AFP to *lacZ* (Fig. 6A and B). The activities of these templates assembled into chromatin in the presence of ML extract (Fig. 6A, lanes 2, 5, and 7) were corrected for effects on basal transcription (lanes 1, 3, and 6) prior to comparison with each other. Removal of nucleotides between −1000 and −541 led to a partial loss of silencing by ML extract (lane 5), a 2.3-fold derepression between this template and the full-length construct. Complete removal of the developmental repressor region to −209 led to a threefold derepression (lane 7). Comparison of these deletion templates as chromatin-free bead-DNA transcribed in HeLa and ML extracts (Fig. 6B) revealed a similar pattern of derepression (compare lanes 5 and 6 to lane 4). In vitro transcription of the deletion templates in HeLa extract (Fig. 6B, lanes 1 to 3) again showed the same slight increase in basal transcription (1.2-fold). Loss of silencing on chromatin-free templates was 5.8-fold upon deletion to −541 and 7.4-fold upon deletion to −209. We therefore focused our analysis of developmental

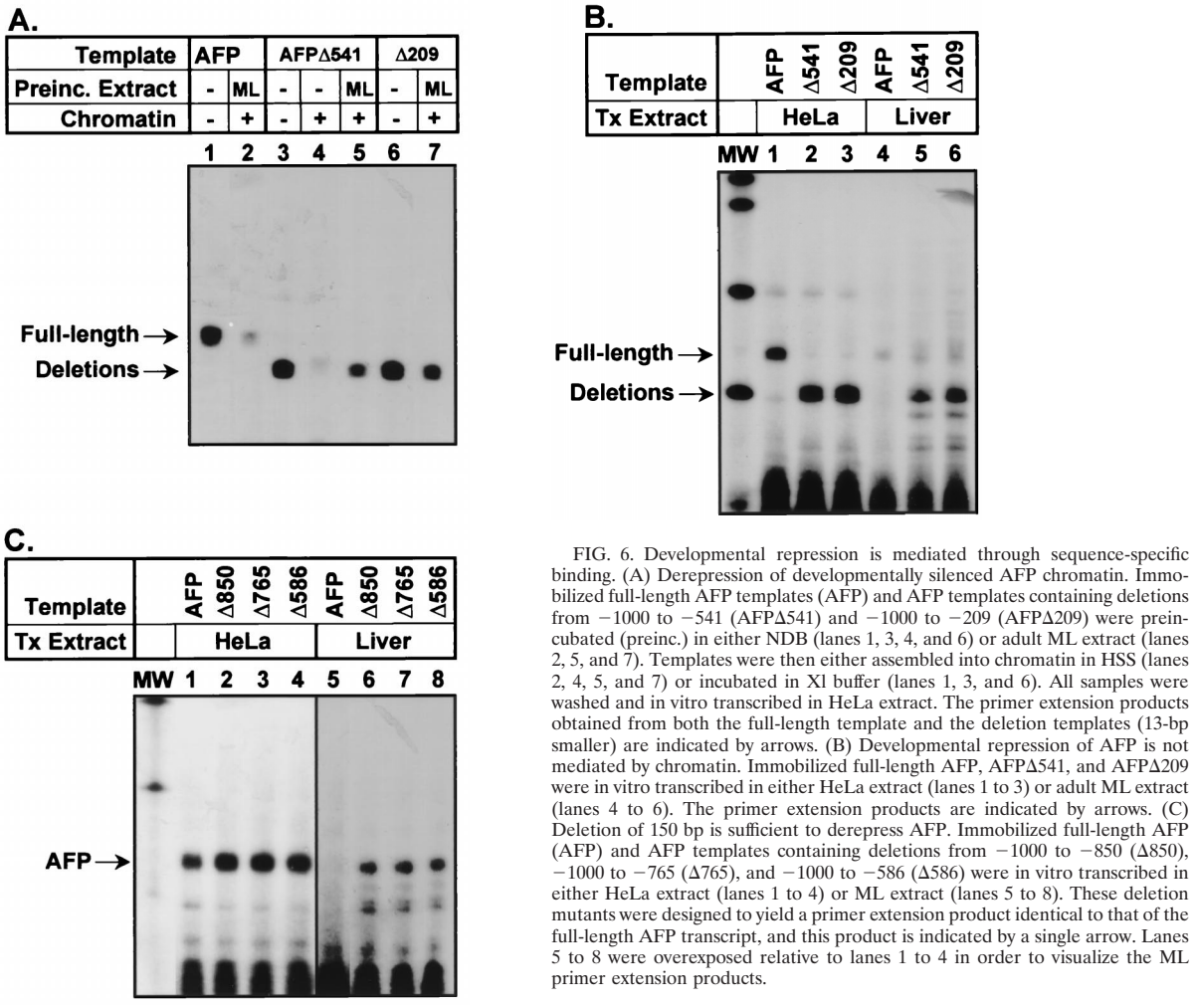

FIG. 6. Developmental repression is mediated through sequence-specific binding. (A) Derepression of developmentally silenced AFP chromatin. Immobilized full-length AFP templates (AFP) and AFP templates containing deletions from −1000 to −541 (AFPΔ541) and −1000 to −209 (AFPΔ209) were preincubated (preinc.) in either NDB (lanes 1, 3, 4, and 6) or adult ML extract (lanes 2, 5, and 7). Templates were then either assembled into chromatin in HSS (lanes 2, 4, 5, and 7) or incubated in Xl buffer (lanes 1, 3, and 6). All samples were washed and in vitro transcribed in HeLa extract. The primer extension products obtained from both the full-length template and the deletion templates (13-bp smaller) are indicated by arrows. (B) Developmental repression of AFP is not mediated by chromatin. Immobilized full-length AFP, AFPΔ541, and AFPΔ209 were in vitro transcribed in either HeLa extract (lanes 1 to 3) or adult ML extract (lanes 4 to 6). The primer extension products are indicated by arrows. (C) Deletion of 150 bp is sufficient to derepress AFP. Immobilized full-length AFP (AFP) and AFP templates containing deletions from −1000 to −850 (Δ850), −1000 to −765 (Δ765), and −1000 to −586 (Δ586) were in vitro transcribed in either HeLa extract (lanes 1 to 4) or ML extract (lanes 5 to 8). These deletion mutants were designed to yield a primer extension product identical to that of the full-length AFP transcript, and this product is indicated by a single arrow. Lanes 5 to 8 were overexposed relative to lanes 1 to 4 in order to visualize the ML primer extension products.

repressor protein interaction on the region between −541 and −1000 of AFP DNA.

Further deletions were constructed by exonuclease digestion from −1000 to −850, to −765, and to −586. Analysis was performed by transcribing the deletion templates in both HeLa and ML extracts in the absence of chromatin assembly (Fig. 6C). Removal of 150 bp to −850 augmented basal transcription slightly relative to the full-length template (1.3-fold; Fig. 6C, compare lanes 1 and 2) and derepressed transcription in ML extracts 7.3-fold (compare lanes 5 and 6). Further deletions to −586 did not result in any further effects on either basal (1.3-fold; Fig. 6C, compare lanes 1 and 4) or derepressed (7.0-fold; compare lanes 5 and 8) transcription. As above, derepression was calculated after correction for effects on basal transcription activity. These analyses show that a developmental repressor DNA-binding element between −1000 and −850 mediates a majority of developmental silencing independently of chromatin assembly. Other *trans*-acting proteins may modulate AFP expression, but clearly a regulatory element for silencing lies within this region.

One candidate repressor protein that binds within this region is the p53 tumor suppressor protein. In vitro analysis revealed that p53 protein binds and excludes hepatocyte nuclear factor 3 activator from an overlapping site between −860 and −833 of AFP. Postnatal repression of AFP expression correlated with induction of p53 protein in 2-week and adult liver nuclear extracts. By transient-transfection analysis with hepatoma and fibroblast cells, we have previously found that p53-mediated repression of AFP expression is tissue specific (35). Studies are under way to identify tissue-specific and ubiquitously expressed repressor proteins that interact with p53 protein and/or bind within the identified 150-bp repressor element to silence AFP expression during development.

DISCUSSION

By establishing a hepatoma-like environment in solid-phase, synthetic nuclei, we have modeled the aberrant activation of AFP gene expression that occurs in vivo during liver tumor formation. We present evidence that replication-mediated activation occurs through a bimodal mechanism: (i) depleting the local concentration of developmental repressor proteins and (ii) facilitating binding of transcription activators to their sites on nucleosomal DNA. These studies indicate that DNA replication, when uncoupled from normal, cellular protein biosynthesis, acts as a driving force in activation of developmentally silenced genes and may amplify tumor marker gene expression during proliferation of hepatoma cells.

DNA replication facilitates activator binding. Reorganization of existing chromatin structure can occur by both replica-

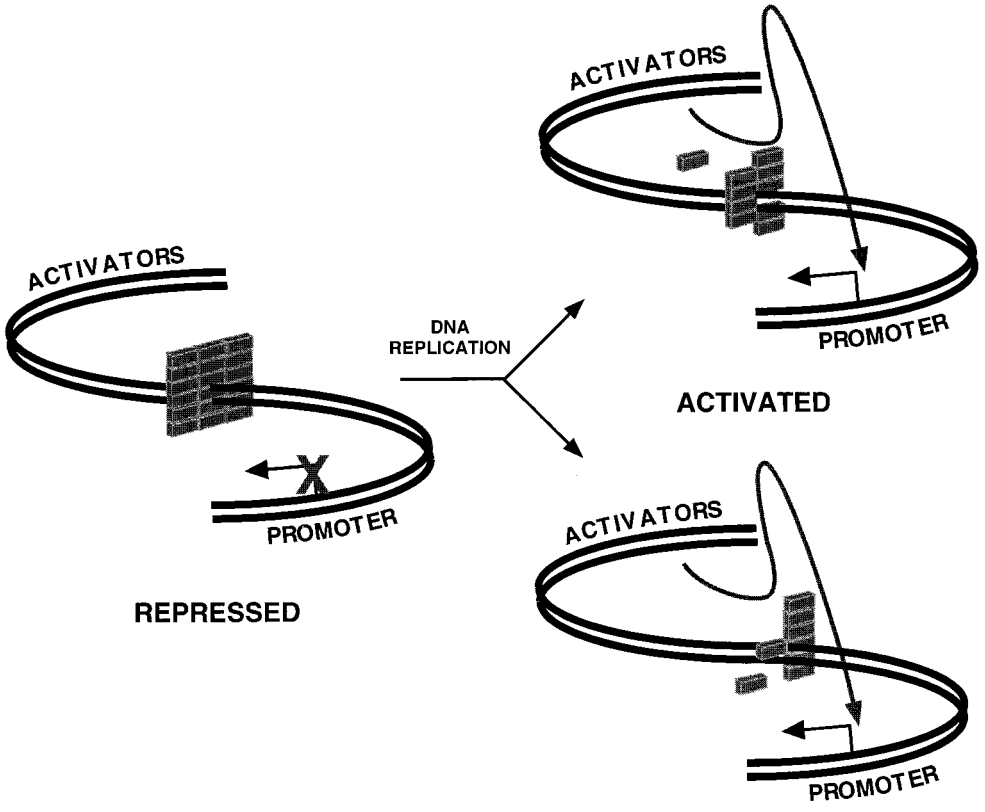

FIG. 7. Model for DNA replication-mediated activation of a developmentally silenced gene. The developmental repressor region of the AFP gene may be bound by a collection of repressor proteins (symbolized by bricks) during postnatal repression. This brick wall prohibits expression of the gene, perhaps by disrupting an upstream activator-promoter communication of an upstream activator(s) with the promoter. Duplication of repressor-binding sites, through either DNA replication or addition of exogenous AFP DNA, effectively depletes the developmental repressors, resulting in incomplete reassembly of the brick wall on the newly replicated DNA. In the presence of an incomplete complex of repressors, activators can now interact with proximal promoter-bound factors in order to initiate transcription.

tion-dependent and -independent mechanisms (e.g., nucleosome remodeling, DNA methylation, and histone modification). It is likely that many layers of control exist to modulate chromatin structure, particularly on genes that must respond rapidly to hormonal fluctuations or environmental challenges (4, 6, 44, 48, 53, 61). Our studies with the AFP gene show that while low levels of replication-independent activation can be observed, DNA replication greatly facilitates derepression of AFP.

DNA replication allows both activators and repressors to gain access to sites normally occluded by nucleosomes (29; reviewed in reference 58). As the majority of transactivators bind with reduced affinity to their sites on nucleosomal DNA versus free DNA (reviewed in references 1 and 60), DNA replication may enable newly synthesized or activated proteins to bind and change gene expression profiles. During tumorigenesis, established gene expression patterns are often disrupted, frequently resulting in reversion to those representative of early development (reviewed in reference 39). The unchecked cell cycling characteristic of tumor cells may facilitate this reprogramming of gene expression patterns.

Parallels for gene regulatory shifts during tumorigenesis are found in regulation at silent mating type loci and telomeres in yeast cells. Relief of telomeric silencing in cases of position effect variegation requires both cell cycle progression and transactivator expression (3), with assembly of silent chromatin occurring as a default. At mating type loci, establishment of the silenced state is an active process that requires passage through S phase (reviewed in references 18 and 37). Thus, although different mechanisms may be involved, reversal of developmental silencing in tumor cells and gene switching at both mating type loci and telomeric regions appear to require progression through the cell cycle.

DNA replication mediates depletion of developmental repressor proteins. Replication of developmentally silenced AFP under limiting ML conditions caused transcription activation. Importantly, replication of chromatin-repressed AFP (no ML present) did not activate AFP expression, demonstrating that the physical process of replication alone is insufficient to activate AFP. We have depicted the repressor complex as a brick wall of multiple component factors which together inhibit activation of the AFP promoter even in the presence of upstream activator proteins (Fig. 7). As would be predicted from this model, repressor (brick) depletion during S phase, at a limiting repressor(s) concentration, may result in incomplete reassembly of repressor complexes (brick walls) on newly replicated DNA. We have shown that repression is recovered by providing additional developmental repressors (bricks) in the form of ML extract during replication. Repression of AFP expression did not occur indirectly by inhibition of DNA replication in solid-phase nuclei, as addition of ML extract does not decrease the relative amount of DNA replication.

Replication timing is linked to cell memory and gene activation. During cell cycle S phase, all genetic material must be faithfully replicated. Numerous studies have demonstrated that chromatin structure is stably propagated from generation to generation (reviewed in reference 56). Passage of the replication fork results in transient disruption of 1 to 2 nucleosomes as well as a majority of DNA-associated *trans*-acting

factors (19, 34, 43), implicating a mechanism for reestablishing protein-DNA contacts postreplication. Models proposed to explain cell memory must consider how expression states, whether active or silent, are both established and maintained. In one such model with particular relevance to our studies, DNA replication timing determines the expression state of a given gene (21). Comparison of numerous tissue-specific and developmental stage-specific genes, including AFP and albumin, with housekeeping genes has revealed a correlation between DNA replication timing and gene transcription, with highly expressed genes replicated early and repressed genes replicated late in the cell cycle (reviewed in references 15 and 26).

Timing of DNA replication has been implicated in silencing at mating type loci and yeast telomeres, in X-chromosome inactivation (reviewed in reference 16), and in tissue-specific expression of certain genes, e.g., globin and *Xenopus* 5S genes (reviewed in references 24 and 28). Mutations in *Saccharomyces cerevisiae* which cause defective silencing at the HMR locus can be suppressed functionally by second-site mutations that increase the time between cell cycle phases, slowing progression through G_1, S, or G_2/M (reviewed in reference 18). One implication of these studies is that a critical concentration of a multicomponent silencing complex of proteins is required, and by lengthening the timing of cell cycle progression, a limiting protein(s) can increase in functional concentration by biosynthesis or posttranslational modification. Similarly, transactivator-mediated derepression of a telomeric *URA3* gene displays strict concentration dependence, suggesting that competition occurs during each cell cycle between the establishment of active and silent states (3).

The present study, as well as dissection of AFP gene regulation in transgenic mouse models, reveals that *trans*-acting factors are required to establish developmental stage-specific silencing (54). Transition between the active fetal state and the silenced postnatal one may rely on competition between repressors and transactivators during DNA replication. Indirect support for this model lies in the brief lag time between postnatal repression of AFP expression and a nearly complete cessation of DNA replication in differentiated hepatocytes (52). In response to liver damage and/or carcinogenesis, differentiated hepatocytes revert in many ways to a fetal phenotype, dividing rapidly and expressing early developmental markers such as AFP. One intriguing possibility is that hepatocyte replication timing is shifted to an early-replicating phase, similar to the developmental switch in replication timing and expression of β-globin genes (32). When challenged by DNA replication early in S phase, the concentration of AFP repressor proteins may be too low to maintain a silenced state, disrupting the balance of differentiation. Using the synthetic nucleus system, it will be possible to introduce various concentrations of a wide spectrum of candidate activators and repressors during DNA replication, with the ultimate goal of reconstituting AFP activation in a defined environment.

The results presented here emphasize the importance of examining gene activation under a range of protein concentrations not only within the context of physiological chromatin, but also during on-going cellular processes such as DNA replication. DNA replication and protein synthesis are strictly regulated in eukaryotic cells (10, 57). By uncoupling these two processes in synthetic nuclei, we have demonstrated that replication in the absence of protein synthesis can have severe effects on the maintenance of established transcription states. Rapid cell cycling, during tumorigenesis or liver regeneration, may therefore upset the balance of transacting factors required for faithful, epigenetic propagation of developmentally regulated expression patterns.

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