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Genome-wide location analysis and expression studies reveal a role for p110 CUX1 in the activation of DNA replication genes

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ABSTRACT

Proteolytic processing of the CUX1 transcription factor generates an isoform, p110 that accelerates entry into S phase. To identify targets of p110 CUX1 that are involved in cell cycle progression, we performed genome-wide location analysis using a promoter microarray. Since there are no antibodies that specifically recognize p110, but not the full-length protein, we expressed physiological levels of a p110 isoform with two tags and purified chromatin by tandem affinity purification (ChAP). Conventional ChIP performed on synchronized populations of cells confirmed that p110 CUX1 is recruited to the promoter of cell cycle-related targets preferentially during S phase. Multiple approaches including silencing RNA (siRNA), transient infection with retroviral vectors, constitutive expression and reporter assays demonstrated that most cell cycle targets are activated whereas a few are repressed or not affected by p110 CUX1. Functional classes that were over-represented among targets included DNA replication initiation. Consistent with this finding, constitutive expression of p110 CUX1 led to a premature and more robust induction of replication genes during cell cycle progression, and stimulated the long-term replication of a plasmid bearing the oriP replicator of Epstein Barr virus (EBV).

INTRODUCTION

CDP/Cux/Cut (CCAAT-displacement protein/cut homeobox) proteins are a family of transcription factors present in all metazoans and involved in the control of proliferation and differentiation (1). The literature in mammals includes a variety of terms, and recently the Human Genome Organization (HUGO) proposed to change from the gene root of CUTL# (CUT-Like #) to CUX#. Thus, the term CUX1 will be used thereafter in the text to designate the human or mouse protein. At least four CUX1 protein isoforms can be expressed as the result of proteolytic processing or transcription initiation at an alternative start site: p200, p150, p110 and p75 (2–5). The full-length protein, p200 CUX1, contains four DNA-binding domains: three Cut repeats (CR1, CR2 and CR3) and a Cut homeodomain (HD) (see Figure 1 for maps) (5). This isoform makes a rapid but unstable interaction with DNA and is responsible for the CCAAT-displacement activity that has been reported in earlier studies (1,6). CUX1 was originally found to function as a transcriptional repressor, but more recent studies showed that the short isoforms repress or activate transcription depending on promoter context (4,7–13). In particular, p110 was found to transactivate a DNA pol α gene reporter in transient transfection assays and to stimulate expression of the endogenous DNA pol α gene following retroviral infection. Using *in vitro* and *in vivo* DNA-binding assays in conjunction with mutated versions of the promoter, a correlation was established between transcriptional stimulation and binding of CUX1 to the promoter (12).

Knockout and transgenic mouse models revealed cell-autonomous as well as non-cell-autonomous phenotypes in multiple organs and tissues (14–18). Cell-based assays have established a role for CUX1 in at least two processes: cell cycle progression and cell motility (19,20). A number of studies demonstrated that CUX1 is regulated in a cell cycle-dependent manner and may have a specific function in S phase. The histone nuclear factor D (HiNF-D), which was later found to include CUX1 as its DNA-binding moiety, was shown to be up-regulated in S phase in normal cells but to be constitutively expressed in various tumor cells (21–25). In NIH3T3 cells, the up-regulation of stable DNA binding at the G1/S transition was shown

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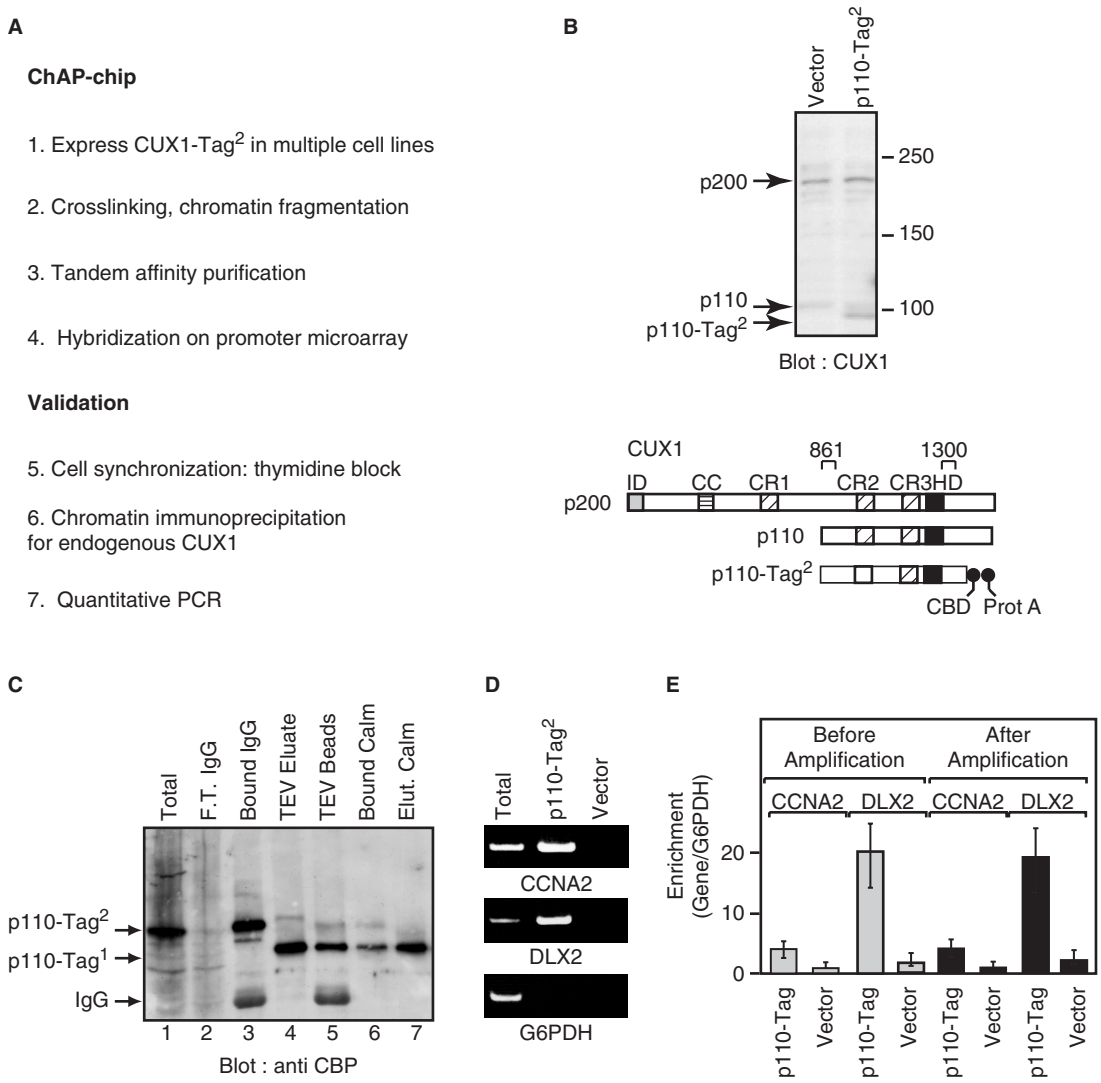

Figure 1. Strategy for the identification of transcriptional targets of p110 CUX1. (**A**) The methodology used to identify gene targets of p110 CUX1 is summarized in a flowchart and is described in the text. (**B**) HeLa cells were infected with a retroviral vector expressing a recombinant p110 CUX1 protein with two tags at its C-terminus. Nuclear extracts were prepared from each population of cells and analyzed by western blot using the 861 and 1300 CUX1 antibodies. Below is a schematic representation of CUX1 proteins with some of the functional domains: ID, inhibitory domain; CC, coiled-coil; CR1, CR2 and CR3, Cut repeat 1, 2 and 3; HD homeodomain; CBD, calmodulin-binding domain; Prot A, protein A. The regions recognized by the 861 and 1300 antibodies are shown. (**C**) Protein samples from each step of the Taptag purification were analyzed by western blot using the anti-calmodulin-binding protein epitope (CBP) Tag antibody. Nuclear extract (lane 1); IgG beads flowthrough (F.T., lane 2); or bound (lane 3); after TEV digestion, cleaved and eluted from IgG beads (lane 4) or still bound to IgG beads (lane 5); bound to calmodulin beads (lane 6) and eluted with EGTA (lane 7). Note that digestion with TEV removes one tag and reduces the size of the recombinant protein. (**D**) Chromatin from Hs578T/p110-Tag^2 and Hs578T/vector cells was submitted to tandem affinity purification and analyzed by PCR using primers specific for the CCNA2, DLX2 and G6PDH gene promoters. Representative data from three independent ChAP experiments are presented. (**E**) The purified chromatin from Hs578T/p110-Tag^2 cells was amplified by ligation-mediated PCR prior to the hybridization. The enrichment level of the CCNA2 and DLX2 gene promoters was measured by quantitative real-time PCR (qPCR) before and after LM-PCR. The results represent the mean ± SD from three independent ChAP experiments and their amplification.

to involve at least two post-translational modifications: dephosphorylation of the Cut homeodomain by the Cdc25A phosphatase, and proteolytic cleavage of p200 CUX1 by a nuclear isoform of cathepsin L (2,4,26). Proteolytic processing of p200 CUX1 at the end of the G1 phase generates the p110 isoform, which contains three DNA-binding domains (CR2, CR3 and HD) and binds stably to DNA (4). Populations of cells stably expressing p110 CUX1 displayed a faster division rate and reached higher saturation density than control cells (20). In various experimental conditions, p110-expressing cells reached the next S phase faster than control cells: following cell synchronization in G0 by growth factor deprivation, synchronization in S phase by double thymidine block treatment, or enrichment in G2 by centrifugal elutriation. In each case, the G1 phase was shortened by 2–4 h (20). Later in the cell cycle, both cyclin A/Cdk2 and cyclin A/Cdk1 were found to interact with p110 CUX1,

but only cyclin A/Cdk1 was able to phosphorylate serine 1237 thereby causing inhibition of DNA binding during the G2 phase (27,28).

In the present study, we performed genome-wide location analysis for p110 CUX1 using chromatin affinity purification (ChAP). Cell cycle targets were validated by conventional ChIP and cell synchronization experiments. Functional annotation revealed an over-representation of genes with various functions required for cell-cycle progression, notably DNA replication and mitosis. In cell-based assays, p110 CUX1 did not affect progression through the G2 and M phases, but was able to stimulate the long-term maintenance of a plasmid carrying the oriP replicator of EBV. Multiple molecular approaches demonstrated that p110 CUX1 transcriptionally activates many genes that play a role in DNA replication. Overall, these results suggest that p110 CUX1 accelerates the start of DNA replication by activating transcription of genes required for DNA replication.

MATERIALS AND METHODS

Cell culture

NIH3T3, HeLa, HEK293 and Hs578T cells were maintained in Dulbecco's modified minimum essential medium (DMEM) supplemented with penicillin–streptomycin, glutamine and 10% fetal bovine serum (FBS) (Gibco) (5% FBS for Hs578T). K562, HEL, Ramos, U266, RPMI8266 cells were grown in RPMI 1640 (Wisent) containing 10% FBS, glutamine and penicillin–streptomycin.

Retroviral infection and stable cell lines

Retroviruses were produced by transfecting 293VSV cells with the pREV/TRE vector either empty or encoding p110 CUX1-Tag^2 (CUX1 aa 612–1336 with protein A and CBP tags inserted at the C-terminus) (Clontech). Preparation of the retroviruses and the eight stable cell lines was done as previously described (20).

Chromatin immunoprecipitation (ChIP)

For ChIP, we used 4×10^8 HeLa cells, either unsynchronized or synchronized by a single thymidine block. Immunoprecipitation of endogenous CUX1 was done using anti CUX1 antibodies 861 and 1300 (4). The nuclei were purified as described in Ref. (29), then lyzed in RIPA-M buffer (10 mM Tris–HCl pH 8, 1 mM EDTA, 0.5 mM EGTA, 150 mM NaCl, 1% Triton X-100, 0.5% DOC, 0.1% SDS, 1 mM PMSF, protease inhibitors) and sonicated on ice to obtain 250- to 800-bp-long DNA fragments. Un-enriched input chromatin was put aside as a control. After preclearing for 1h and incubation with antibodies overnight, immunocomplexes were washed 3 times each in wash buffer I (20 mM Tris–HCl pH 8, 2 mM EDTA, 2 mM EGTA, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.2% SDS), wash buffer II (20 mM Tris–HCl pH 9, 2 mM EDTA, 2 mM EGTA, 500 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS), wash buffer III (50 mM Tris–HCl pH 7.5, 2 mM EDTA, 1 mM EGTA, 0.5 M LiCl, 1% NP-40, 0.7% DOC,) and then washed once in Tris–EDTA. Cross-linked DNA was eluted with 1% SDS, 10 mM Tris–HCl pH 8, 10 mM EDTA at 65°C for 30 min. After reversal of formaldehyde cross-linking, chipped DNAs were treated with RNase A and Proteinase K.

Enrichment calculation

ChIP experiments were analyzed by real-time PCR using the FastStart DNA Master SYBR Green kit (Roche). Fold enrichment of target promoters was calculated in two steps. First, the fold enrichment for each target gene was determined relative to a negative control (G6PDH promoter) and was expressed as percentage of input. Second, the specific enrichment was calculated by dividing the enrichment level in the sample obtained with CUX1 antibody by the (non-specific) enrichment level in the sample obtained with no antibody.

Chromatin affinity purification (ChAP)

For each cell line, the ChAP-chip procedure was performed independently at least 3 times, each time comparing the p110-Tag^2 and the vector alone stable line. A total of 3 to 6×10^8 cells were used for each purification. Total chromatin was obtained and sonicated as described above and a fraction was put aside as un-enriched input control. Stably expressed recombinant p110-Tag^2 protein was purified by the Taptag purification method with some modifications (30). The cross-linked sonicated DNA was prepared in the same way as in the ChIP. The IgG matrix bound p110-Tag^2/DNA were washed with above-mentioned wash buffers I, II and III, and then TEV buffer (10mM Tris–HCl (pH 8.0), 100 mM NaCl, 0.1% TX-100, 0.5 mM EDTA, 10% glycerol, 1 mM DTT). After TEV protease digestion, the released protein–DNA complexes were purified by affinity chromatography on calmodulin beads in the presence of calcium and then eluted with EGTA. After de-cross-linking, samples were treated with RNase A and Proteinase K. In all experiments, each purification step was monitored by immunoblotting with polyclonal antibodies against the calmodulin-binding domain epitope tag or against CUX1. A detailed protocol for ChAP-chip is provided as a Word file in the Supplementary Data.

Probe generation and microarray hybridization

For each cell line, microarray hybridizations were performed 3 times using independently obtained ChAP-enriched and input DNAs. The generation of labeled DNAs from individual ChAP samples was performed following the protocol of linker-mediated PCR (LM-PCR) as detailed previously (31). Briefly, ChAPped DNAs and input DNA were blunted, ligated to a unidirectional linker and amplified by PCR for 24 cycles to generate a sufficient amount of DNA. In all experiments, the enrichment level of the DLX2 gene promoter before and after LM-PCR amplification was verified by real-time PCR. ChAP and input DNAs were fluorescently labeled with Cy5 fluorophore and Cy3 fluorophore, respectively, by using BioPrime Array CGH genomic labeling kit following the manufacturer's instructions (Invitrogen).

Prior to hybridization, microarray slides were incubated in a blocking solution, 1.6% succinic anhydride in 1-methyl-2-pyrrolidinone, for 20 min at RT. After washing, labeled DNAs were added to the hybridization buffer (25% Formamide, 5× SSC, 0.1% SDS, 0.2% BSA, 0.4 μg/μl of human Cot-1 DNA, 0.8 μg/μl of yeast tRNA) and hybridized at 55°C for 20 h. The slides were washed once with 2× SSC, 0.1% SDS for 15 min, twice 2 min with 0.1× SSC, 0.1% SDS, twice 1 min 0.1× SSC and then spun dried. Hybridized slides were scanned with an Axon 4000b scanner and the acquired images were analyzed with the software GenePix Pro, Version 4.1.

Microarray design

A microarray containing 19k human promoters was generated as reported (32). In brief, the regions ranging from 800 bp upstream and 200 bp downstream of the transcription start sites from 18 660 human genes were amplified by PCR and QC tested and applied on an Poly-L-lysine glass slides together with 188 controls located in exons and far from any known genes.

Microarray data analysis

The analysis of the ChAP-chip results was done as described (33). Promoters were considered 'bound' when the binding *P*-value in the error model was <0.005. Functional categories were established using the online DAVID (Database for Annotation, Visualization and Integrated Discovery) software, available at: http://david.niaid.nih.gov/david. Classes with a *P*-value <0.02 were considered over-represented.

Promoter sequence analysis

Promoter sequences were determined by extending the microarray probe by 250 bp both upstream and downstream. Occurrence of ATCRAT and CCAAT sequences in CUX1 targets ($n = 52$) and non-targets ($n = 55$), as defined by ChAP-Chip results, were determined using the MacVector software.

Western and Southwestern assays

Western blotting was performed as previously described (4). Southwestern blotting was performed as previously described using a double-stranded oligonucleotide probe containing the CUX1 consensus-binding site: CGATA TCGAT (34).

Pixel quantification was done with the Scion Image 1.63 software.

Luciferase assay

Luciferase reporters were constructed as follows: PCR amplification was performed to obtain fragments of genomic DNA containing the sequence present on the microarray plus at least 250 bp on either side. The fragments were cloned into the luciferase reporter vector, pGL3 (Promega). Assays were performed as previously described (12).

Expression analysis of p110 CUX1 target genes

Total RNA was extracted and RT-PCR was carried out as previously described (19). Expression levels of the genes were determined by real-time PCR using G6PDH as an internal control. As for infection analysis, Hs578T cells were infected with an empty vector or a retrovirus expressing p110 CUX1 and total RNA was extracted 24 h post-infection. For siRNA knockdown experiments, NIH3T3 cells were transfected with either CUX1-specific siRNA or a scrambled RNA by using Lipofectmine2000 reagent (InVitrogen) and total RNA was extracted 48 h post-transfection. Serum starvation experiments using NIH3T3 stably expressing p110 CUX1 or carrying the empty vector were performed as described previously (19). Cells were harvested at indicated time points.

Cell synchronization and FACS analysis

For ChIP, HeLa cells were synchronized by a single thymidine block (2 mM thymidine, 16 h). Double thymidine block and serum starvation/stimulation were performed as previously described (20). Cell cycle analysis was done by FACS and the cell cycle distribution was determined using the Watson model and the FlowJo™ software (20).

EBV plasmid long-term maintenance assays

Hs578T cells stably expressing p110 CUX1 or carrying the empty vector were transfected with 1 μg of pc3oriPE plasmid by using the Lipofectamin 2000 reagent and cells were grown in 800 μg/ml of G418 for up to 10 days (35). Drug-resistant colonies from long-term maintenance assay were visualized by staining with crystal violet. Quantification was done with the Scion Image 1.63 software.

RESULTS

Establishment and validation of the chromatin affinity purification (ChAP) method

Our goal was to identify targets that are bound specifically by the p110 isoform of CUX1. As p110 CUX1 is generated by proteolytic processing of p200 CUX1, the entire primary sequence of p110 is contained within p200 and, in spite of our attempts, there is currently no CUX1 antibody that can recognize p110 at the exclusion of p200. We therefore developed an alternate strategy to identify the p110-specific targets. We established populations of cells stably expressing physiological levels of a p110 CUX1 protein with two epitope tags at its C-terminus, p110-Tag^2, and we purified chromatin by tandem affinity purification (TAP) (36). We refer to this procedure as chromatin affinity purification (ChAP) and the subsequent microarray analysis as ChAP-chip (Figure 1A, see also the ChAP-chip protocol in Supplementary Data). To ensure that the recombinant p110-Tag^2 protein would be expressed at moderate level, we employed the pRevTRE retroviral vector (Clontech), which contains the minimal CMV promoter with a tetracycline-responsive element. Retrovirally infected cells typically carry only one or a few copies of the viral DNA. In the absence

of a tetracycline-responsive transactivator, basal expression from the pRevTRE vector was previously shown to be very low (37–39). Indeed, p110-Tag^2 was expressed at relatively low level in our population of HeLa/p110-Tag^2 cells (Figure 1B). Similar results were obtained with the other populations in the absence of tetracycline (data not shown). In addition, we isolated six independent HeLa/p110-Tag^2 clones and verified that expression did not vary significantly among individual clones (data not shown).

The p110-Tag^2 protein was followed by immunoblotting through each step of the tandem affinity purification protocol (Figure 1C). To verify the specificity of the method, we tested for the presence of two known targets of CUX1, CCNA2 (cyclin A2) and DLX2, in chromatin samples that had been purified by affinity chromatography from cells expressing p110-Tag^2 or from control cells carrying the empty retroviral vector [(20) and unpublished data]. Whereas the CCNA2 and DLX2 genes were present in the chromatin purified from p110-Tag^2 cells, these genes could not be detected in the chromatin from control cells (Figure 1D). Moreover, real-time qPCR was performed for all samples to ensure that the enrichment level was not changed following LM-PCR amplification of the purified DNA (Figure 1E).

Strategy to identify targets of p110 CUX1

We performed location analysis using a panel of eight human cell lines of various cell types: breast tumor (Hs578T), cervix carcinoma (HeLa), kidney epithelial (HEK293), myeloid leukemia (K562, HEL) and B cell lymphoma (Ramos, U266, RPMI8266). For each cell line, hybridization experiments were done in triplicate using three independent ChAP-enriched and input DNA samples (8 cell lines $\times$ 3 hybridizations = 24). ChAP-enriched DNA regions associated with p110 CUX1 were purified and labeled with Cy5 dye. Equal amounts of Cy5-labeled ChAP products and Cy3-labeled input DNA samples were co-hybridized onto a human promoter microarray. We utilized a genomic DNA microarray containing the region spanning 800 bp upstream and 200 bp downstream of transcription start sites of 18 660 human genes (32).

To determine the *P*-value threshold that would be used, we performed standard PCR analysis on 37 randomly selected targets using ChAP-enriched chromatin obtained from Hs578T cells expressing the p110-Tag^2 protein or carrying the empty vector (Figure 2). All targets with a *P*-value under 4×10^{-3} were confirmed, whereas 5 out of 6 targets with *P*-values between 4 and 5×10^{-3} were confirmed (Figure 2). Based on these experiments, we used $P < 0.005$ because our estimated false-positive rate was less than 3% using this threshold. Importantly, none of the randomly tested targets was found to be present in the chromatin that was purified from cells carrying the empty vector (Figure 2). However, as an additional control, hybridization of the promoter array was performed in triplicate with chromatin purified from a cell line carrying the empty vector. These experiments led to the identification of a total of 19 genes with a *P*-value under 0.005 (Supplementary Table 5). These genes were removed from our list of putative targets.

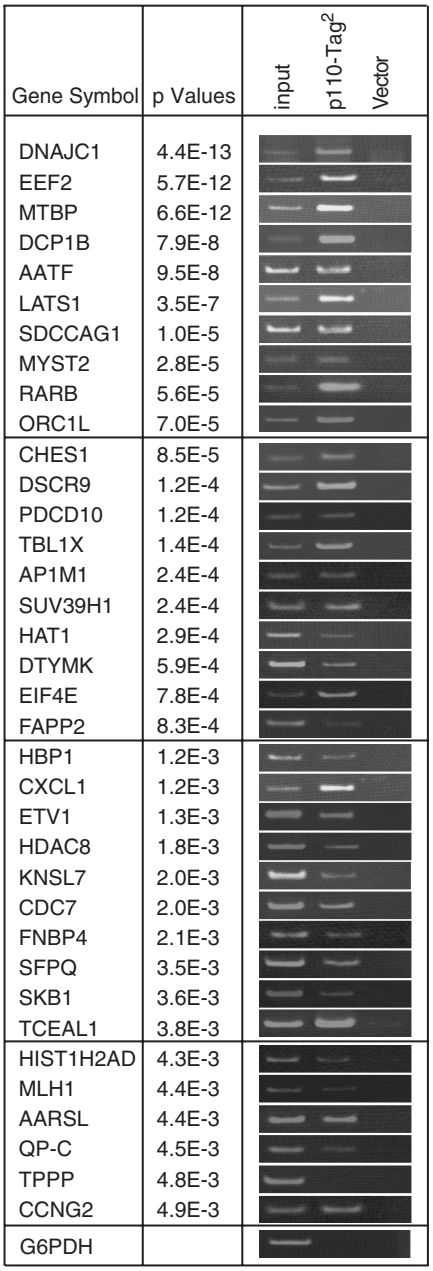

Figure 2. PCR analysis of target promoters with various *P*-values. Chromatin from Hs578T/p110-Tag^2 and Hs578T/vector cells was submitted to tandem affinity purification and analyzed by PCR using primers specific for each promoter. Input DNA (0.1%) was used as control. Note that the region to be amplified was chosen to be approximately in the middle of the sequence spotted on the location array. The *P*-values were obtained from microarray hybridizations performed in triplicate and comparing three independent ChAP-enriched and input DNA samples.

Scanning ChAP analysis was performed on three loci, AATF, RARB and SUV39H1, to verify where p110 CUX1 was recruited within the locus. In each case, a signal was observed in the region immediately upstream of the transcription start site and nowhere else (Figure 3).

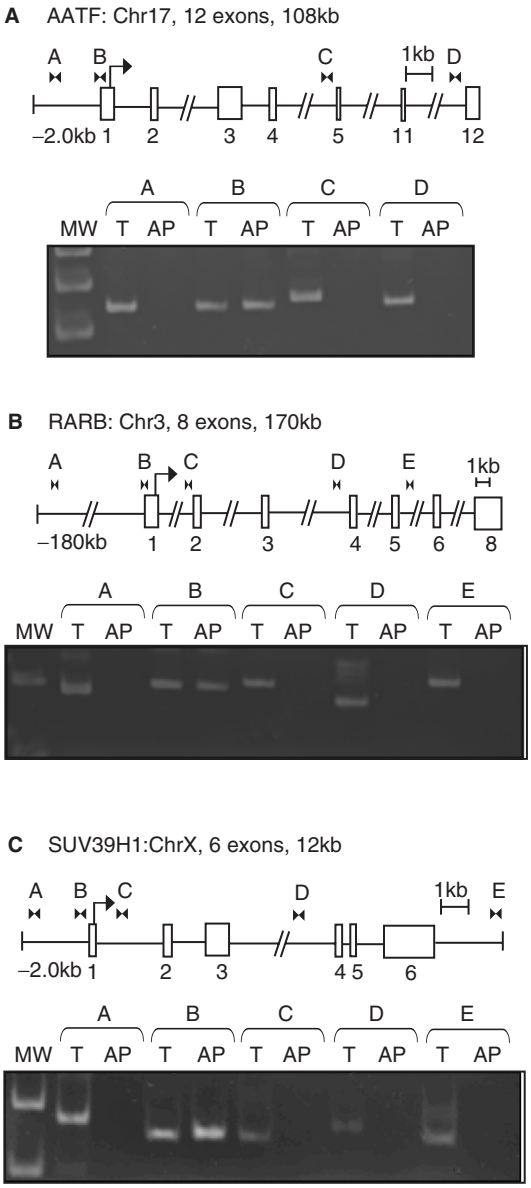

Figure 3. Scanning ChAP analysis of the AAFF, RARB and SUV39H1 genes. Chromatin from Hs578T/p110-Tag[2] cells was submitted to tandem affinity purification and analyzed by PCR using primers specific for different regions of the following loci: AAFF (**A**), RARB (**B**) and SUV39H1 (**C**). Templates for the PCR reactions were 0.1% total input DNA (T) or ChAP-purified DNA (AP). Exons are numbered and the transcription start sites are indicated by arrows. The positions of amplified fragments are indicated over the maps and primer sequences are given in Supplementary Table 7.

We conclude that p110 CUX1 is recruited specifically to the promoter region of these three genes.

Over-representation of cell cycle functions among targets of p110 CUX1

To verify whether specific cellular functions were over-represented among the putative target genes of p110 CUX1, a single list was compiled from the eight cell lines (Supplementary Table 6). A hierarchy of functional categories was established using the online DAVID software.

Table 1. Functional classes among transcriptional targets of p110 CUX1

Biological Process (level 5)			
Function	All[a]	Bound[b]	*P*-value
Mitosis	1.04%	2.05%	0.0075
DNA replication initiation	0.26%	0.82%	0.0083
Mismatch repair	0.20%	0.68%	0.0106
Antigen processing via MHC class I	0.15%	0.68%	0.0028

Genes that were bound by p110 CUX1 were compared with all genes present on the microarray by using a web-based functional annotation tool, DAVID. Over-represented functions that are statistically significant (cutoff *P*-value <0.02) are shown.
[a]'All' designates all genes that were spotted on the microarray.
[b]'Bound' designates the genes enriched in the ChAP-chip experiments with p110.

Among the functional classes most over-represented were mitosis, DNA replication initiation, mismatch repair and antigen processing via MHC class I (Table 1). The list of genes that play a role in proliferation and cell cycle progression is shown in Table 2.

Conventional ChIP confirms the recruitment of CUX1 to the promoters of cell cycle targets in S phase

CUX1 should bind a cell cycle target in all cell lines independently of the tissue-type of origin. Yet we noticed that not all targets displayed a *P*-value below 0.005 in all eight cell lines (Supplementary Table 6). To explain this discrepancy, we considered the possibility that the recruitment of CUX1 to cell cycle gene promoters occurred primarily in S phase as previously demonstrated (12). As a consequence, the signals in microarray hybridizations would be expected to be relatively weak since only a small fraction of cells are in S phase in a population of asynchronously proliferating cells. We set out to test this hypothesis and, at the same time, verify whether the endogenous CUX1 protein was binding to the promoters of cell cycle genes. Conventional ChIP was performed using CUX1 antibodies and HeLa cells either unsynchronized or enriched in S phase by the thymidine block procedure. FACS analysis of the cell cycle distribution indicated that the proportion of cells in S phase was increased almost 3-fold following the thymidine block (Figure 4A). Although the expression level of p110 CUX1 was constant, the intrinsic DNA-binding affinity of p110 CUX1 was clearly increased as judged from Southwestern blotting (Figure 4B, lanes 1–2 and 3–4). Pixel quantification of three independent experiments, using the Scion Image software, estimated to 2.7 ± 0.54-fold the increase in DNA binding in cells treated with thymidine. These observations are in agreement with what was reported in previous studies (4,12,28,40). Quantitative PCR analysis was performed on 49 gene promoters using ChIP-enriched chromatin from the two populations of cells. We observed a moderate enrichment of targets in the unsynchronized cells (Table 2, column 4). However, the enrichment was consistently much higher when the chromatin was taken from a population of cells enriched in S phase (Table 2, column 5). These results suggest that in most cases

Table 2. Transcriptional targets of p110 CUX1 that play a role in cell cycle

Function	Gene symbol	Gene description	ChIP/qPCR: Fold/total	
			Unsync.	Thymidine
Cell cycle, S phase	CCNA2	Cyclin A2	1.6	5.1
	CDC25A	Cell division cycle 25A	2.5	7.5
Cell cycle, G2/M	CDC25B	Cell division cycle 25B	2.1	5.6
	CDC25C	Cell division cycle 25C	0.7	3.2
	MAD2L1	MAD2 mitotic arrest deficient-like 1 (yeast)	1.2	3.7
Cell cycle, M Phase	ANAPC4	Anaphase-promoting complex subunit 4	2.9	4.8
	APC10	Anaphase-promoting complex subunit 10	1.8	2.6
	EML4	Echinoderm microtubule-associated protein-like 4	2.6	4.0
	FNBP4	Formin-binding protein 4	1.7	14.1
	FZR1	Fzr1 protein	3.7	24.6
	KIF11	Kinesin family member 11	2.0	8.0
	KNSL7	Kinesin-like 7	1.8	4.7
	KNTC1	Kinetochore-associated 1	0.7	7.9
	LATS1	Large tumor suppressor 1	5.0	11.8
	NUMA1	Nuclear mitotic apparatus protein 1	1.9	2.2
	PRC1	Protein regulator of cytokinesis 1	3.6	5.5
	SKB1	SKB1 homolog (*S. pombe*)	1.8	4.7
	SMC4L1	SMC4 structural maintenance of chromosomes 4-like 1	3.5	8.0
	TOPK	T-LAK cell-originated protein kinase	3.5	3.4
Cell cycle, meiosis	STAG2	Stromal antigen 2	9.2	2.5
DNA replication	CDC45L	CDC45 cell division cycle 45-like (*S. cerevisiae*)	0.8	9.0
	CDC7	CDC7 cell division cycle 7 (*S. cerevisiae*)	2.1	5.4
	CHAF1A	Chromatin ssembly factor 1, subunit A (p150)	1.4	5.9
	DNTT	Deoxynucleotidyltransferase, terminal	1.2	4.0
	DTYMK	Deoxythymidylate kinase (thymidylate kinase)	2.1	10.3
	MCM3	MCM3 minichromosome maintenance deficient 3	2.8	13.0
	MCM7	MCM7 minichromosome maintenance deficient 7	1.9	5.3
	MYST2	MYST histone acetyltransferase 2	2.9	14.6
	ORC1L	Origin recognition complex, subunit 1-like (yeast)	2.0	7.2
	ORC3L	Origin recognition complex, subunit 3-like (yeast)	1.6	2.1
	POLA	Polymerase (DNA-directed), alpha	3.0	13.2
	POLA2	Polymerase (DNA-directed), alpha (70 kDa)	7.2	21.3
	POLD2	Polymerase (DNA-directed), delta 2,	0.6	6.3
	POLD3	Polymerase (DNA-directed), delta 3, accessory subunit	2.9	17.0
	RPA3	Replication protein A3, 14 kDa	1.9	7.9
Proliferation	CCNH	Cyclin H	2.4	8.1
	EGF	Epidermal growth factor	2.0	4.5
	MVP	Major vault protein	3.5	13.6
	PURA	Purine-rich element-binding protein A	0.9	5.1
Repair, checkpoint	ATR	Ataxia telangiectasia-related	1.9	3.9
	CCNG2	Cyclin G2	2.9	13.1
	CHES1	Checkpoint suppressor 1	2.2	22.4
	MTBP	Mdm2 binding protein	2.6	3.2
	TP53	Tumor protein p53 (Li-Fraumeni syndrome)	1.9	6.0
Repair, homologous	RAD51	RAD51 homolog (RecA homolog, *E. coli*)	2.9	17.0
Repair, mismatch	MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	1.1	5.2
	MSH6	MutS homolog 6 (*E. coli*)	3.5	13.6
	PMS1	PMS1 postmeiotic segregation increased 1	2.9	6.3
	PMS2L5	Postmeiotic segregation increased 2-like 5	1.2	3.7

The targets are organized in functional classes (column 1). Indicated are the gene symbols (column 2), the enrichment fold in conventional ChIP assays performed with HeLa cells, either unsynchronized (column 4) or submitted to a thymidine block (column 5). Enrichment of target promoters was calculated using the G6PDH locus as a reference and is shown relative to the chipped DNA obtained by immunoprecipitation with no antibody.

p110 CUX1 binds to the promoter of cell cycle genes primarily during S phase. Importantly, all 49 cell cycle targets were validated by conventional ChIP in cells enriched in S phase.

Sequence analysis of target promoters

A motif-finding algorithm was used to identify sequences that are over-represented in the promoters of targets genes. The CUX1 consensus-binding site, ATCRAT (R = A or G), was the most frequent motif found in the p110-bound promoters (Table 3) (41). Further inspection of promoter sequences did not reveal a particular arrangement of these motifs relative to the transcription start site as they appear to be randomly distributed. In contrast to the ATCRAT sequence, the CCAAT motif and the consensus-binding site for the Cut repeats 1 and 2, CRAT-CRAT, were not over-represented

within the promoters of target genes (Table 2) (6). Similarly, other sequences proposed to represent binding sites for CUX1 were not present more often in the promoter of target genes (42–44).

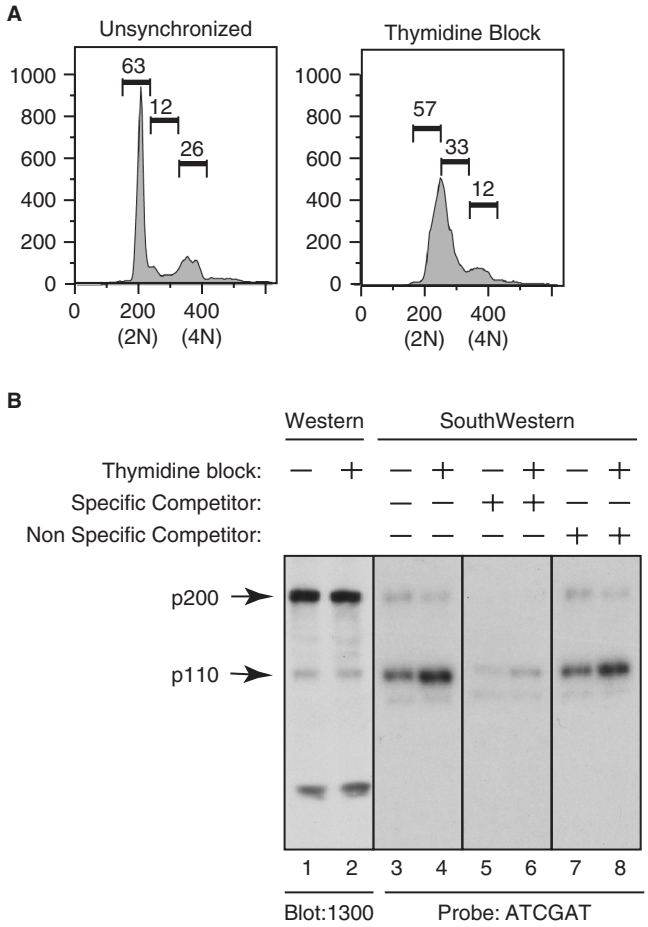

Figure 4. Synchronization of HeLa cells using the thymidine block procedure. Exponentially growing HeLa cells were submitted to a single thymidine block. (**A**) Nuclei were stained with propidium iodide and DNA content was determined by fluorescence-activated cell sorting (FACS) analysis. Cell cycle profiles were analyzed using the Watson model and the FlowJo™ software. The numbers above the brackets indicate the percentage of cells with 2N DNA content (G0/G1 cells), between 2N and 4N DNA content (S phase cells) and 4N DNA content (G2/M cells). (**B**) Nuclear extracts were prepared from synchronized and unsynchronized cells and were submitted to western and Southwestern blot analysis using CUX1 1300 antibodies (Figure 1) and oligonucleotides containing a consensus-binding site for p110 CUX1: ATCGAT. Competition experiments were performed in the presence of an excess amount (1000×) of unlabeled specific or unrelated oligonucleotides (third and fourth panels).

p110 CUX1 activates transcription of most cell cycle-related target genes

To verify the effect of p110 CUX1 on the expression of cell cycle-related target genes, Hs578T breast tumor cells were infected with a retrovirus expressing p110 CUX1 or with an empty retrovirus. This cell line was chosen because in previous studies using reporter assays it displayed the greatest fold difference upon expression of various CUX1 isoforms (12). We postulate, therefore, that the Hs578T cells express non-limiting amounts of the factors and co-factors that cooperate with CUX1 in transcriptional regulation. After 24 h, RNA was purified from the two populations of infected cells and the expression of a subset of genes in each functional class was analyzed by quantitative PCR. In most cases, gene expression was stimulated in cells that express p110 CUX1, but with some notable exceptions. Expression of cyclin H and $p21^{WAF1/CKI1}$ was reduced, whereas that of MYST2 was not significantly affected (Figure 5 and Table 4). Assuming that changes in gene expression are a direct consequence of the interaction of p110 CUX1 with the promoter of these genes (see Discussion section), these results indicate that the

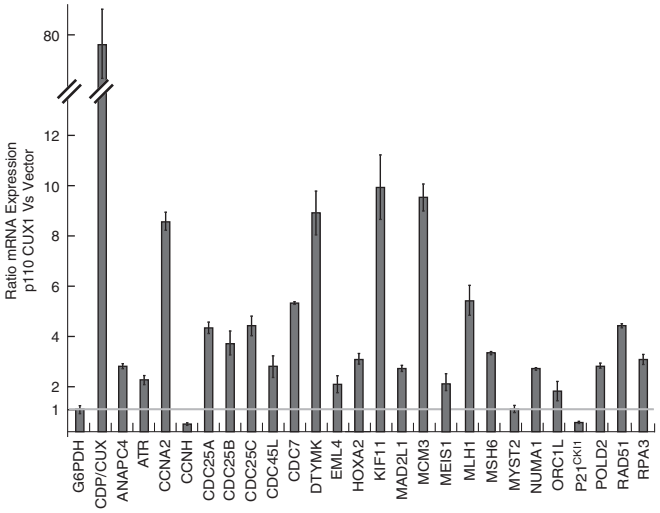

Figure 5. Expression of most targets is stimulated following transient expression of p110 CUX1. Hs578T cells were infected with a retrovirus expressing p110 or with an empty retrovirus. RNA was prepared 24 h post-infection. mRNA expression was measured by quantitative real-time PCR using primer pairs specific for CUX1 and for each target. G6PDH levels were used to normalize the samples. The values are the mean of three measurements and error bars represent standard deviation. The fold difference in expression and *P*-values are presented in Table 4.

Table 3. 'ATCRAT' motif is over-represented in CUX1 cell cycle target genes

Motifs		ATCRAT	CCAAT	CRAT/CRAT	CRAT/GYTA
Percentage of promoters with motif(s)	Targets	52%**	90%	17%	15%
	Non-targets	13%	87%	15%	11%
Number of motifs per promoter	Targets	0.71**	2.40	0.21	0.15
	Non-targets	0.15	1.91	0.16	0.11

The promoter regions of target (Table 2) and 50 non-target genes were analyzed for the presence of putative CUX1-binding sites using the MacVector software. Promoter regions were defined as the sequences that were spotted on the microarray plus 250 bp on each side. R = A or G; Y = T or C. The significance of the difference between Targets and Non-Targets for each motif was determined by Student's t-test. $^{**}P = 0.01$.

regulatory effect of p110 CUX1 is dependent on promoter context: it functions as a transcriptional activator of most cell cycle-related target genes, but acts as a repressor of some specific targets. These results confirm and extend previous observations showing that CUX1 represses the $p21^{WAF1/CKI1}$ gene but activates the cyclin A2 and DNA pol alpha genes (1,12,45,46).

The immediate promoter region is sufficient for the activation by p110 CUX1

To verify whether the 1-Kbp region around the transcription start site was sufficient to enable regulation by p110 CUX1, promoter sequences from 15 target genes were cloned into a luciferase reporter plasmid. Promoter fragments included the sequences that were spotted on the microarray plus ~250 bp on either side. The two genes that were repressed in the infection assays, CCNH and $p21^{WAF1/CKI1}$, were also repressed in the reporter assay (Figure 6 and Table 4). Similarly, genes that were activated in the infection assays were also activated in the reporter assay, with the exception of HOXA2 (Figure 6 and Table 4). We conclude that the genomic regions identified through the ChAP-chip procedure in most cases contain *cis*-acting sequences that are sufficient for the regulation by p110 CUX1. The fact that some promoters were repressed while others were activated reinforces the notion that the regulatory effect of p110 CUX1 is promoter dependent.

Table 4. Regulatory interactions between p110 CUX1 and cell cycle targets

Gene symbol	Infection assay		Reporter assay	
	Fold	*P*-value	Fold	*P*-value
ANAPC4	2.9	2.0*E*−04		
ATR	2.3	1.9*E*−02		
CCNA2	9.3	1.5*E*−03	9.0	2.5*E*−03
CCNH	0.34	2.0*E*−02	0.6	1.3*E*−02
CDC25A	4.6	8.0*E*−04	14.1	9.7*E*−03
CDC25B	3.9	5.0*E*−04	8.7	5.7*E*−03
CDC25C	4.7	2.7*E*−03	2.9	1.4*E*−02
CDC45L	2.9	4.8*E*−03		
CDC7	5.7	1.4*E*−03		
DTYMK	9.7	1.2*E*−02	5.4	9.3*E*−03
EML4	2.1	8.9*E*−03		
HOXA2	3.2	1.5*E*−02	1.0	3.9*E*−01
KIF11	10.0	3.8*E*−03		
MAD2L1	2.8	1.6*E*−02		
MCM3	9.7	1.0*E*−04	5.2	1.0*E*−04
MEIS1	2.2	5.7*E*−03	6.3	1.1*E*−02
MLH1	5.8	2.9*E*−02		
MSH6	3.5	1.4*E*−03	8.3	1.8*E*−02
MYST2	1.0	1.6*E*−01	4.3	5.0*E*−04
NUMA1	2.8	5.2*E*−03		
ORC1L	1.8	6.1*E*−03	4.4	2.0*E*−03
P21	0.4	3.6*E*−02	0.2	1.0*E*−04
POLD2	2.9	1.1*E*−03		
RAD51	4.7	1.8*E*−03	2.9	7.5*E*−03
RPA3	3.2	1.0*E*−03	5.7	1.5*E*−02

The table lists the fold difference in expression level of endogenous genes following the infection of Hs578T cells with a p110 CUX1 expression vector (column 2), the *P*-value of the difference in expression (column 3), the fold difference observed in reporter assays using plasmids carrying the promoter sequences of various targets (column 4), and the *P*-value of the difference in reporter assays (column 5). Two rows are highlighted to stress the fact that two targets were repressed, while most targets were activated.

p110 CUX1 stimulates DNA replication

Two classes of genes were clearly dominant among the validated cell cycle targets of p110 CUX1: genes that play a role in mitosis and those involved in DNA replication (Table 2). In previous studies, p110 CUX1 was found to accelerate entry into S phase, however, no obvious change had been noticed later in the cell cycle (2,20). We re-investigated this issue, but no change in the progression through the G2 and M phases were observed in two cell lines stably expressing p110 CUX1 (Supplementary Figure 10, NIH3T3 and NMuMG). We then tested whether p110 CUX1 could stimulate DNA replication. Three approaches were used to test this hypothesis. First, we measured the expression of DNA replication gene targets following the inhibition of CUX1 expression by siRNA in NIH3T3 cells. As a control, cells were treated in a similar manner with a scrambled RNA sequence. The expression of all 15 DNA replication targets was reduced, in some cases drastically, concomitantly with the decrease in CUX1 expression (Figure 7). These results indicate that CUX1 is required for the maximal expression of DNA replication genes.

Next, we monitored the expression of DNA replication gene targets following serum starvation/re-stimulation of NIH3T3 cells stably expressing p110 CUX1 or carrying the empty vector. Gene expression was measured by qPCR at 6 (G1), 18 (G1/S) and 24 (S) h after serum addition as well as in unsynchronized cells. The cell cycle distribution of cells was determined by FACS analysis following staining of the DNA with propidium iodide (Figure 8). The expression of most DNA replication targets was higher in the p110 CUX1 expressing cells at the three time points, 6, 18 and 24 h, yet it was still

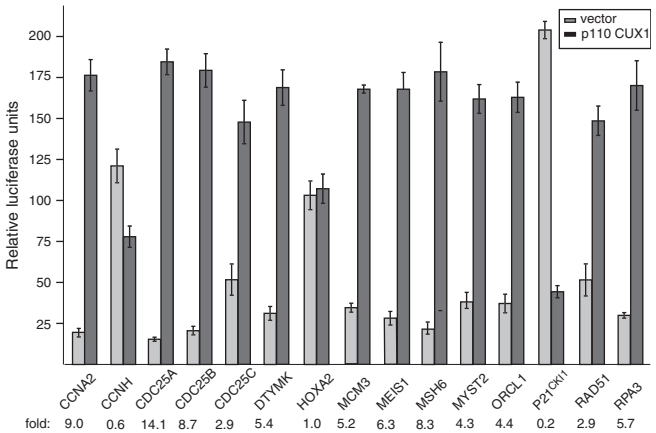

Figure 6. Luciferase reporter assays. The promoter regions of 15 target genes were cloned into a luciferase reporter plasmid. Hs578T cells were transfected with each reporter plasmid together with a vector expressing p110 CUX1 or with an empty vector (vector). The values are the mean of three independent experiments and error bars represent standard deviation. The fold difference in expression and *P*-values are presented in Table 4.

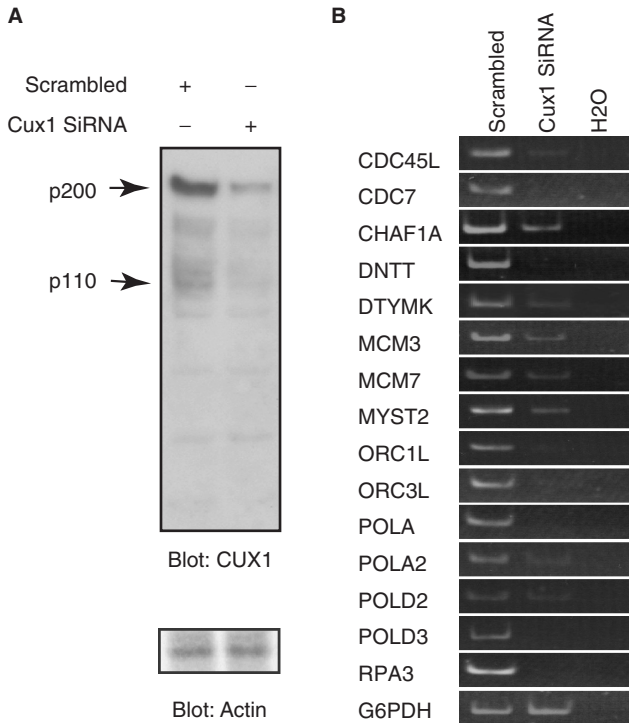

Figure 7. Knockdown of CUX1 using siRNA causes a decrease in the expression of DNA replication target genes. NIH3T3 cells were transfected with CUX1-specific siRNA or, as a control, a scrambled RNA. Cells were harvested 48 h post-transfection, nuclear protein extracts and total RNA were prepared. CUX1 protein expression was analyzed by immunoblotting with the 861 CUX1 antibodies (left panel). The expression of target genes was analyzed by RT-PCR using G6PDH as a control as indicated.

regulated in a cell cycle-dependent manner. Thus, the absolute level of expression for most genes was the highest at 18 h. The implications of these results in terms of transcriptional regulation will be discussed below.

Finally, we performed a long-term *in vivo* DNA replication assay using a plasmid bearing the oriP replicator of Epstein Barr virus (EBV). Replication of oriP containing plasmids depends on cellular factors and occurs no more than once per cell cycle (47). More efficient replication and maintenance of the plasmid were observed in Hs578T cells constitutively expressing p110 CUX1 than in control cells carrying an empty vector (Figure 9). In contrast, when a non-replicating plasmid was transfected, no significant difference was observed in the number of resistant colonies between the two populations (data not shown). Altogether, the results show that p110 CUX1 is required for maximal expression of DNA replication genes, and that constitutive expression of p110 CUX1 not only can increase the expression of genes involved in DNA replication but also can stimulate the replication and maintenance of a plasmid that is controlled by replication licensing.

DISCUSSION

The goal of this study was to identify the transcription targets of p110 CUX1 that are involved in cell cycle progression. To circumvent the problems due to the fact that CUX1 antibodies all recognize the full-length p200 isoform, we expressed a p110-Tag[2] recombinant protein and purified the chromatin by tandem affinity chromatography (Figure 1). To validate this approach, chromatin was purified in parallel from cells carrying the empty expression vector and we verified the enrichment of two known targets of p110 CUX1 and later, of 36 randomly chosen targets with *P*-values under 0.005 (Figures 1D and 2). In no case did we observe the enrichment of a known or of a putative target in the chromatin obtained from cells carrying the vector only. To validate specifically the cell cycle targets identified in location array analysis, we carried out conventional chromatin immunoprecipitation with CUX1 antibodies, using a population of cells enriched in S phase as well as a population of asynchronously proliferating cells, and we performed quantitative real-time PCR for each of the 49 cell cycle targets (Table 2). Overall, the results demonstrated that the method of ChAP-chip indeed is able to identify genuine targets with a low rate of false positive. We stress, however, that this method is valid as long as one is able to express the recombinant protein at physiological levels. In our experience, most commonly used expression vectors are not adequate for this type of procedure. Only the Rev-TRE retroviral vector, in the absence of a tetracycline-responsive activator, generated a reasonably low level of expression that was comparable to that of endogenous p110 CUX1 (Figure 1B and data not shown).

Our cell synchronization experiment revealed that CUX1 was recruited to the promoter of cell cycle target genes more efficiently during S phase (Table 2). Interestingly, the proportion of cells in S phase was increased around 3-fold following the thymidine block. Yet, for many targets the enrichment was much higher than in unsynchronized cells, up to 24-fold. The fact that the enrichment was considerably weaker in asynchronously proliferating cells helps explain why many targets genes were originally identified in only one or two cell lines in microarray analysis, as opposed to being present in all cell lines as expected for a cell cycle gene (Table 2). The signal for any of these genes would be expected to be relatively weak since most cells in a population of asynchronously proliferating cells are in G1.

The size and the complexity of CUX1 proteins have contributed to the difficulty in identifying optimal binding sites for CUX1. One advantage of ChIP and ChAP technologies is that it enables the unbiased identification of genomic binding sites. The *in vivo* binding sequences can then be scanned for the presence of binding motifs that were established using *in vitro* binding assays. Among all putative CUX1-binding sites reported in the literature, only one, the ATCRAT motif, was found to be over-represented among targeted sequences as compared to non-targeted sequences (Table 3). This finding confirmed that the ATCRAT motif indeed is a preferred binding site for p110 CUX1 *in vivo*. Yet, approximately only one half of the targeted promoters contained this consensus-binding site. This proportion is lower than what was reported for some transcription factors, but this finding is not exceptional (32). In a recent study, the E2F1 consensus site was found within 12% of sequences bound

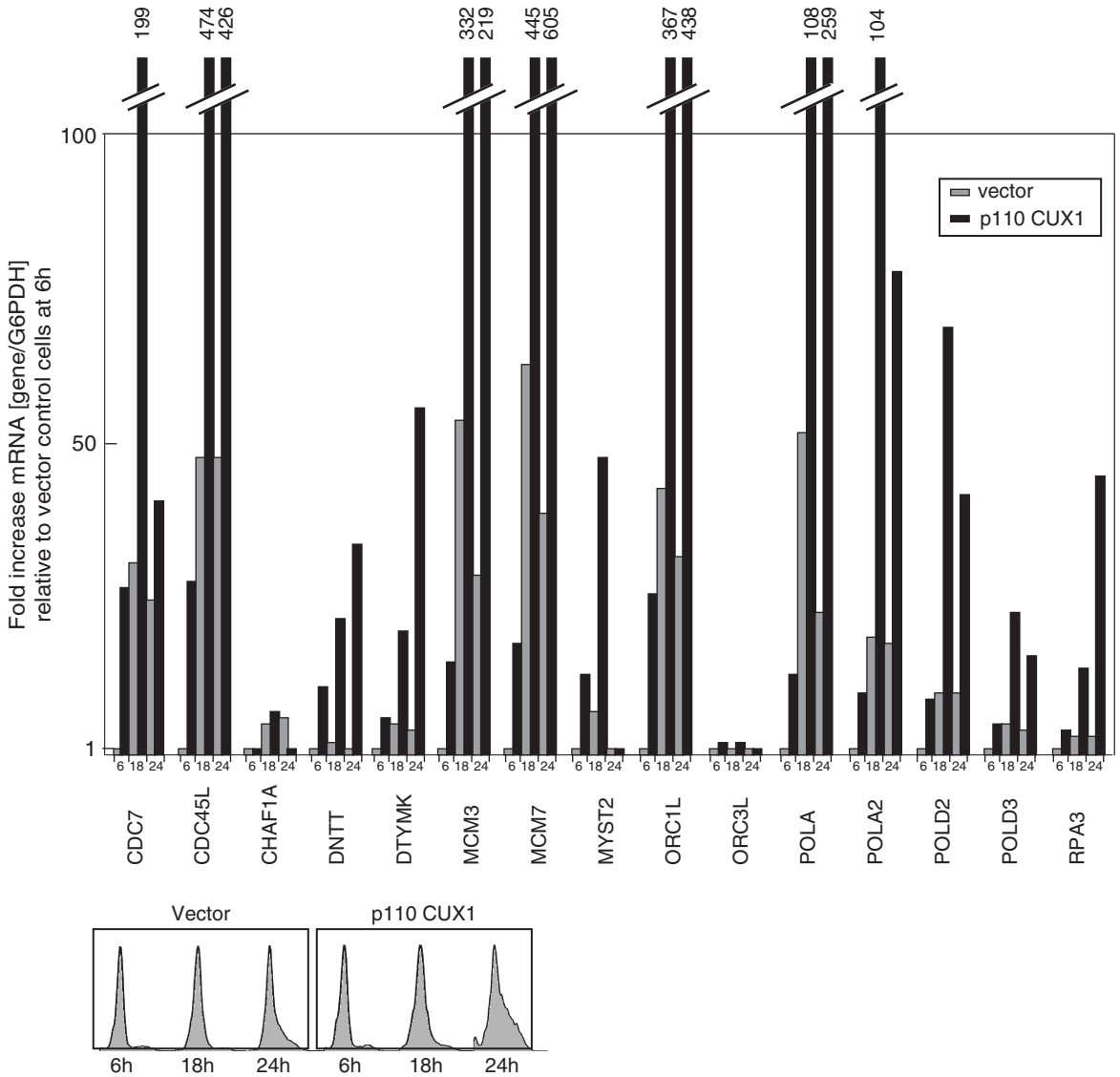

Figure 8. Constitutive expression of p110 CUX1 leads to higher expression of DNA replication target genes. RNA was prepared from NIH3T3 cells stably expressing p110 CUX1 or carrying the empty vector and maintained in various culture conditions. Cells were cultured asynchronously or were serum-starved for 3 days and then re-stimulated with 10% serum for 6, 18 and 24 h. The expression of DNA replication target genes was analyzed by RT-PCR using G6PDH as a control as indicated. For each gene, expression level at 6 h in vector control cells was arbitrarily given a value of 1 and expression levels at other times were expressed relative to this value.

in vivo (48). We conclude that the recruitment of CUX1 to promoters *in vivo* can be driven, like E2F1, from its interaction with low-affinity binding sites and, to a large extent, from its interaction with other proteins.

A large fraction of genes regulated by p110 CUX1 code for proteins that play a role in mitosis, but we did not find a difference in the duration of the G2 and M phases in cells stably expressing this transcription factor (Supplementary Figure 10). This unexpected result again is reminiscent of similar findings obtained with the E2F transcription factors both in expression profiling and ChIP-chip assays (31,49,50). Yet, to our knowledge, forced expression of an E2F factor has not been reported to affect progression through the G2 and M phases. The failure of these transcription factors to induce a faster progression at the end of the cell cycle probably reflects the fact that post-transcriptional mechanisms play a crucial role in the regulation of the G2/M transition and the progression through M phase. Moreover, the G2-specific transcription of some genes was previously shown to result from the periodic occupation of a repressor element (51). Nevertheless, it appears that the transcriptional program that leads to the execution of mitosis begins to be orchestrated during S phase. We can envision that the start of DNA replication triggers the transcriptional activation of genes whose products will be required for mitosis and cytokinesis. This does not necessarily mean that these genes will be turned on immediately, but that transcriptional complexes must begin to be assembled on their promoters. Future studies should verify whether E2F and p110 CUX1 cooperate in the regulation of these genes.

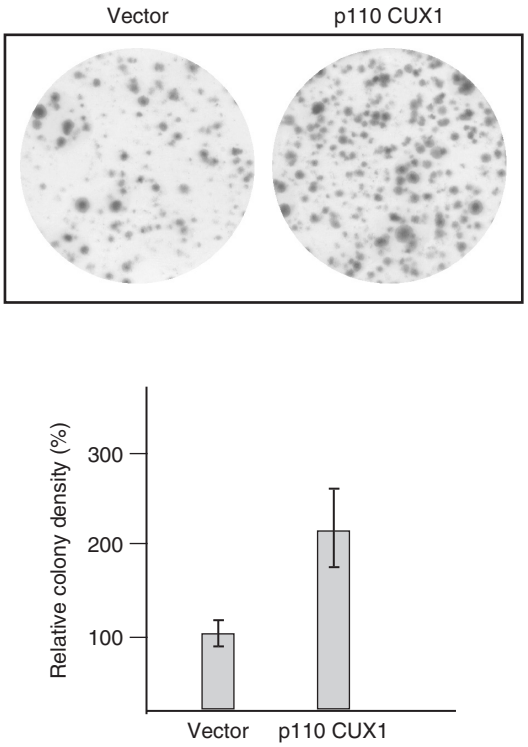

Figure 9. Constitutive expression of p110 CUX1 stimulates the long-term replications and maintenance of an EBV replicon. The pc3oriPE plasmid that carries oriP, coding sequences for EBNA1 and the resistance for G418 was transfected into Hs578T/p110 and Hs578T/vector cells. After transfection, cells were grown under G418 selection for 10 days and the resistant colonies were visualized by staining with crystal violet. The images are representative of results obtained in three separate experiments. Shown in the histogram is the colony density as measured using the Scion Image 1.63 software.

The effect of p110 CUX1 on the expression of cell cycle-related targets was investigated using four distinct approaches: gene expression was measured in cells transiently infected with a retroviral vector expressing p110 CUX1, in cells treated with CUX1 siRNA, in cells stably expressing p110 CUX1, and in reporter assays. The results from all these approaches concur to indicate that most cell cycle targets are activated by p110 CUX1, while a few are repressed or are unaffected. These findings are in contrast with the bulk of the literature on CUX1 (1). The reason for this is that earlier studies focused on the full-length CUX1 protein which functions exclusively as a repressor. We previously demonstrated that the removal of the N-terminal half of the protein, which contains an auto-inhibitory domain and the Cut repeat 1, has important consequences on the DNA binding and regulatory properties of the protein (4,12,13). Yet, despite our progress, we have yet to uncover the molecular basis for the dual role of p110 CUX1 as an activator and a repressor. In this respect, no obvious clue was provided from the analysis of promoter sequences of two genes that are repressed, cyclin H and $p21^{WAF1/CKI1}$, and of genes that are activated. We surmise that the regulatory effect of p110 CUX1 is determined by the specific complex it forms with other partners on distinct promoters.

Our results demonstrated that p110 CUX1 could stimulate the long-term replication of a plasmid carrying the oriP replicator from EBV (Figure 9). We believe the mechanism at play is the early and more pronounced transcriptional activation of genes that code for products required for DNA replication. Indeed, expression of many DNA replication genes was higher in cells expressing p110 CUX1 than in control cells (Figure 8). Interestingly, the absolute level of DNA replication gene expression was highest at 18 h however the fold increase relative to the vector only cells was already important at 6 h. A number of observations can be made from the results of this experiment. First, higher expression of p110 CUX1 is not sufficient to render constitutive the expression of DNA replication genes. Other regulatory events are required for their maximal transcription, some that activate the DNA-binding ability of CUX1 and others that certainly affect factors that cooperate with CUX1. Second, whether at the peak of gene expression or earlier in G1, p110 CUX1 is obviously a limiting factor in the transcriptional activation of DNA replication genes. Third, it is not clear that the striking peak in gene expression at 18 h is responsible for the accelerated S phase entry in cells that constitutively express p110 CUX1. We think it is likely that the activation of gene expression earlier in G1 has important consequences. In other words, the quantitative difference at 18 h may be functionally less relevant than the fact that the transcriptional program that leads to S phase entry is initiated earlier in G1. At the molecular level, we envision that the constitutive expression of p110 CUX1 permits the precocious assembly of transcriptional complexes on the promoter of cell cycle genes. Future studies should attempt to determine whether all or only a small subset of direct targets contribute to stimulation of DNA replication and the acceleration of S phase entry.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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