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Transcription Factor Binding and Induced Transcription Alter Chromosomal c-*myc* Replicator Activity

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The observation that transcriptionally active genes generally replicate early in S phase and observations of the interaction between transcription factors and replication proteins support the thesis that promoter elements may have a role in DNA replication. To test the relationship between transcription and replication we constructed HeLa cell lines in which inducible green fluorescent protein (GFP)-encoding genes replaced the proximal ~820-bp promoter region of the c-*myc* gene. Without the presence of an inducer, basal expression occurred from the GFP gene in either orientation and origin activity was restored to the mutant c-*myc* replicator. In contrast, replication initiation was repressed upon induction of transcription. When basal or induced transcription complexes were slowed by the presence of α-amanitin, origin activity depended on the orientation of the transcription unit. To test mechanistically whether basal transcription or transcription factor binding was sufficient for replication rescue by the uninduced GFP genes, a GAL4p binding cassette was used to replace all regulatory sequences within ~1,400 bp 5′ to the c-*myc* gene. In these cells, expression of a CREB-GAL4 fusion protein restored replication origin activity. These results suggest that transcription factor binding can enhance replication origin activity and that high levels of expression or the persistence of transcription complexes can repress it.

Models for DNA replication in eukaryotes derive significantly from studies of *Saccharomyces cerevisiae* and *Xenopus laevis* systems, in which prereplication complexes (pre-RCs) containing the hexameric origin recognition complex ORC, Cdc6, and the minichromosome maintenance proteins are activated by the cell cycle-regulated kinases Cdc7/Dbf4 and cyclin-dependent kinases to unwind DNA and load DNA polymerases for the initiation of DNA synthesis (reviewed in reference 8). Although replication origins in higher organisms do not generally display the conserved size, sequence, or structure of replicators in *S. cerevisiae*, this overall series of events appears to be conserved in fission yeast and metazoan somatic cells, and the demonstration that chromosomal regions involved in the initiation of replication can promote replication when transferred to ectopic chromosomal sites provides genetic evidence for the existence of defined replicator elements that can control replication initiation in the chromosomes of multicellular organisms (1, 2, 4, 51, 52).

Replication origins are frequently found upstream of eucaryotic transcription units (reviewed in reference 57), consistent with the suggestion that transcription and replication may be oriented to coordinate transcription and replication fork movement (13, 27, 50). Transcribed sequences are generally replicated earlier in S phase in the cells where they are expressed than in cells where they are not (22, 76), possibly as a result of the modification of chromatin structure by transcription factors (48). However, several investigators report positive (11, 19, 21, 25, 34, 36, 47, 53, 66, 86) or negative (11, 43, 53) effects of transcription factors on DNA replication, either directly through the binding of replication proteins or indirectly via modulation of chromatin structure. Alternatively, it has been suggested that cells may replicate active sequences prior to gene expression as a mechanism to avoid disruption of pre-RCs by the passage of the transcriptional machinery (12, 28, 57).

Multiple transcription factor binding sites and transcription regulatory elements are located in the 2.4-kb region upstream of the human c-*myc* gene (32, 33, 58). Our laboratory initially reported that replication initiates in this region (46, 54, 55), and Vassilev and Johnson first used quantitative PCR (Q-PCR) to define the replication initiation zone (83). Subsequent reports have confirmed these observations (21, 80, 82, 84) and shown that plasmids containing the 2.4-kb fragment of the c-*myc* 5′ flanking DNA replicate autonomously in vivo and in vitro (10, 38, 54–56, 64, 82). In *Xenopus* and mouse cells, replication initiates at the corresponding locations relative to the c-*myc* genes (29). When moved to an ectopic site in the HeLa genome, the 2.4-kb human c-*myc* core origin fragment promotes replication initiation in the sequences flanking its insertion site, providing genetic evidence for its function as a chromosomal replicator (51, 52). The 2.4-kb core origin fragment harbors an in vivo DNA unwinding element (DUE) (7, 23, 58) flanked by three matches to the *S. cerevisiae* 11-bp autonomously replicating sequence consensus sequence. Deletion of the DUE or of fragments comprising putative transcription factor binding sites eliminates chromosomal replicator activity (51).

In a direct test of the effect of transcription on the activity of the c-*myc* replicator in a chromosomal environment we used a genomic integration approach to analyze replicator constructs in which inducible green fluorescent protein (GFP) genes re-

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placed the c-*myc* promoter and transcription factor binding site region. To avoid variable chromosomal position effects, clonal cell lines were constructed that contained each c-*myc* replicator integrant at the same genomic location (51, 52). In stably transfected cells the chromatin of the ectopic c-*myc* integrants was hyperacetylated. While deletion of the transcription factor binding region eliminated origin activity, fusion of the inactive c-*myc* replicator upstream region to the GFP transcription unit restored replicator activity to levels above those of the wild-type c-*myc* sequence. Upon the induction of transcription no large-scale changes in chromatin structure were detected, but origin activity was repressed back to roughly the level of the wild-type replicator. These observations indicate that the structure of the replicator upon transcription factor binding or basal expression is qualitatively different than that seen following induction. When a CREB-GAL4 fusion protein was targeted to an integrated c-*myc* replicator construct from which all promoter elements within ~1,400 bp 5′ of the c-*myc* gene had been deleted, origin activity was strongly enhanced. Mechanistically, these results imply that transcription factor binding can enhance replication initiation independently of its effect on transcription.

MATERIALS AND METHODS

Cell culture and DNA isolation. HeLa cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 10% newborn calf serum (Invitrogen) and 10 μg of gentamicin/ml in a humidified 5% CO_2 atmosphere at 37°C. GFP expression was induced with doxycycline (1 μM) for 6 or 24 h. To arrest transcription, uninduced cells or cells treated 6 h earlier with doxycycline were treated with 50 μg of α-amanitin/ml for 1 h.

Plasmid constructs and transfections. Construction of the pHyg.FRT.TK acceptor site plasmid containing the 48-bp FLP recombinase target (FRT) sequence, generation of the acceptor cell line HeLa/406, and targeted integration with FLP recombinase have been described previously (51, 52). The 1.7-kb enhanced GFP (EGFP) transcription unit containing the tetracycline response element (TRE), miniCMV promoter, EGFP open reading frame, and simian virus 40 polyadenylation sequence was amplified from pBI-EGFP (Clontech) (nucleotides [nt] 3882 to 459) by use of primers with ApaI restriction sites. The 426-bp TRE promoter contains seven *tetO* 18-mers fused to a mini-cytomegalovirus (mini-CMV) promoter. The mini-CMV promoter TATA box is located 33 bp 3′ to the TRE. The 3′ ApaI-ApaI fragment (nt 1542 to 2367; nt 1 = HindIII cleavage site) of the 2.4 kb c-*myc* sequence was replaced with a TRE-GFP transcription unit in either orientation. In the TRE-GFP forward orientation, this places the mini-CMV promoter in the same position relative to the 5′ c-*myc* sequences as the endogenous c-*myc* P_0 promoter. The pFRT.TRE-GFP plasmid was derived from pFRT.myc.TRE-GFP by partial digestion with ApaI and complete digestion with Pin1 to remove all but the most 5′ 79 bp of the c-*myc* replicator. The ends were filled by T4 DNA polymerase and blunt-end ligated. The construct with the inducible transcription units transcribed in the same direction as the endogenous c-*myc* gene is referred to as exhibiting forward orientation. To develop the doxycycline-inducible cell lines the forward and reverse clonal cell lines were stably transfected with the pTet-ON plasmid expressing the tetracycline-controlled transactivator and the pTet-tTs suppressor plasmid (Clontech). Cells that were transiently transfected with pFRT.myc.TRE-GFP plasmids had stably integrated pTet-ON and pTet-tTs.

To construct the pFRT.myc.3′Δ1420-GAL4 cell line the following sequence was cloned between Spe1 (nt 929) and ApaI (nt 2367) restriction sites: GCTG AGCGGAAGACTCTCCTCCGAATTCCGGAAGACTCTCCTCCGCGGGG AAGACTCTCCTCCGGAGCGGCCGCCATCGGAGCACTGTCCTCCGAA CTTCCATCGGAGCACTGTCCTCCGAACTT. All constructs were verified by DNA sequencing.

The CREB-GAL4 fusion protein was expressed from pCRG4-11, a most generous gift of Patrick Quinn (Pennsylvania State University). In pCRG4-11, CREB amino acids 1 to 277 are fused to GAL4p amino acids 4 to 147. Replacement of the CREB DNA binding and dimerization domain with the GAL4p DNA binding domain maintains the CREB activation domain in its native conformation (69). GAL4p was expressed using the same vector from which the CREB cDNA had been deleted. Expression of CREB-GAL4p and GAL4p was confirmed by Western blotting using anti-GAL4 antibody (Abcam). The efficiency of transient transfections was normalized by cotransfection with a β-galactosidase expression plasmid or by immunostaining with anti-GAL4 antibody and quantitation by flow cytometry (CREB-GAL4, GAL4).

RNA isolation and cDNA preparation. Total RNA was isolated using an RNeasy kit (QIAGEN). Reverse transcriptase reactions were performed using 1 μg of total RNA, random hexamers, and a Thermoscript RT-PCR system (Gibco-BRL). Prior to reverse transcription, RNA preparations were treated with RNase-free DNase (Ambion) to remove DNA, as assessed by real-time PCR. After reverse transcription, cDNA was quantitated by real-time PCR. For each cDNA preparation the amplification values were normalized to the abundance of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA. In control reactions using cDNA from HeLa cells, the PCR signal from GFP was undetectable. At least six to eight PCR quantitations were performed on each of multiple RNA preparations.

Southern blot hybridization and diagnostic PCR. A total of 10 μg of genomic DNA was digested with either HindIII or EcoRI (Gibco-BRL) overnight, electrophoresed in 0.8% agarose gels in 1× Tris-acetate-EDTA, and blot transferred to Hybond N^+ membranes (Amersham) following standard protocols (73). After blotting, the filters were UV cross-linked (Stratalinker) and prehybridized for 2 h at 42°C in 50% formamide–10% dextran sulfate–0.5% sodium dodecyl sulfate–1 M NaCl–1 mM EDTA (pH 8.0)–50 μg of denatured salmon sperm DNA/ml. Hybridization was performed with the prehybridization solution without salmon sperm DNA overnight at 42°C by use of the 407-bp NcoI-SmaI fragment from pFRT.myc (Neo probe), the 1.5-kb EcoRI-XbaI fragment from pHyg.FRT.TK (thymidine kinase [TK] probe), or the 756-bp EcoRI-XbaI fragment from pHyg-.FRT.TK (hygromycin resistance gene [Hyg] probe). For Northern blot hybridizations, 20 μg of total RNA was used. RNA was electrophoresed and blotted to Hybond N^+ membrane (Amersham) following standard protocols (5). A segment of the human β-actin gene (nt 3200 to 3320) and the 750-bp BamHI fragment from pFRT.myc.TET-GFP were gel purified and used as probes with the actin and EGFP genes, respectively. A total of 50 ng of genomic DNA was used for PCR with primer sets specific for either the unoccupied acceptor construct (primers 1 and 2) or the acceptor construct after integration of the donor plasmids (primers 1 and 3) as described previously (51, 52).

Chromatin immunoprecipitation (ChIP). Cells were cross-linked according to the protocol of Ritzi et al. (70) with minor modifications. Cross-linked chromatin was resuspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA) and sonicated (Branson sonifier cell disrupter 200) (50% duty cycle; 10-s pulses; 7 pulses with 1-min intervals). Chromatin was digested with 0.1 U of micrococcal nuclease (Sigma) per 100 μg of nucleoprotein at 37°C for 5 min to yield fragments of 150 to 400 bp. Nucleoprotein (250 μg) was diluted with 11× NET (550 mM Tris-Cl [pH 7.4], 1.65 M NaCl, 5.5 mM EDTA, 5.5% NP-40) to a final concentration of 1× NET. Anti-acetyl histone H4 antibody (Upstate Biotechnology) or anti-RNA polymerase II (Pol II) antibody (N-20; Santa Cruz Biotechnology) was used for immunoprecipitation. Washing of the antibody complex and purification of the precipitated DNA was carried out according to the method of Schepers et al. (75), and immunoprecipitated DNA was quantitated by real-time PCR.

PCR and analysis of nascent DNA. Asynchronous populations of cells were used for nascent DNA isolation as described previously (41). Nascent DNA was size fractionated in 1.25% alkaline agarose gels and neutralized, and fragments of 1 to 2 kb were extracted using a QIAquick gel extraction kit (QIAGEN). Nascent DNA abundance was measured by real-time Q-PCR using SYBR Green in an Applied Biosystems 5700 or 7000 instrument. Primers specific for the FRT acceptor site did not amplify HeLa DNA. Q-PCR data were normalized against the signal from the low-nascent-strand-abundance site STS-54.8 in the human β-globin locus (21, 41) amplified from an equal amount of the same nascent DNA in the same 96-well tray by use of the same reagents. Standard curves were developed individually for each primer set using known amounts of the same preparation of 406/pFRT.myc (c-*myc* wild-type) cell genomic DNA. The data were compiled from at least three separate nascent DNA preparations for each cell line and three or more independent PCR amplifications of each nascent DNA preparation. Under the conditions of our experiments, >95% of the measurements are expected to fall within 30% of the indicated mean (61). The primers shown in Table 1 and other primers described previously (51) were used for PCR amplification.

Chromatin structure analysis. Micrococcal nuclease digestion assays of the ectopic c-*myc* locus in HeLa cells were performed as described previously (44). Nuclei were mildly digested (0.06 U/A_{260} or 0.1 U/A_{260}) with MNase (Sigma) at room temperature for 4 min. DNA was isolated and digested to completion with NcoI. Electrophoresis, blotting, and Southern hybridization of the digested DNA were done as described above.

TABLE 1. PCR primers

Assay	Primer	
	Upper	Lower
STS-UV Q-PCR	TGCCTGACTGCGTTAGCAAT	CAAACCCTAAAACGGCCAAA
STS-DV Q-PCR	TCCCTGGCTCCCCTCCT	CATAAGTGCGGCGACGATA
GFP Q-RT-PCR	CAACAGCCACAACGTCTATATCATG	ATGTTGTGGCGGATCTTGAAGT
GAPDH Q-RT-PCR	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
Endogenous c-*myc* Q-PCR	CCAAACCAAATACCCATCACCTT	TGAAAGCAAGACACTGGATTTATTG
	GGCTTGGCGGGAAAAAGA	CCCGAAAACCGGCTTTTATAC
RNA Pol II ChlP Q-PCR		
Promoter	CGTTTAGTGAACCGTCAGATCG	CCGGTGTCTTCTATGGAGGTCA
GFP 1	ACCGCATCGAGCTGAAGG	CCAGCTTGTGCCCCAGG
GFP 2	AGCAAAGACCCCAACGAGAA	GGCGGCGGTCACGAA
SV40[a]	GGTATGGCTGATTATGATCCTGC	GGACCTTGCACAGATAGCGTG

[a] SV40, simian virus 40.

RESULTS

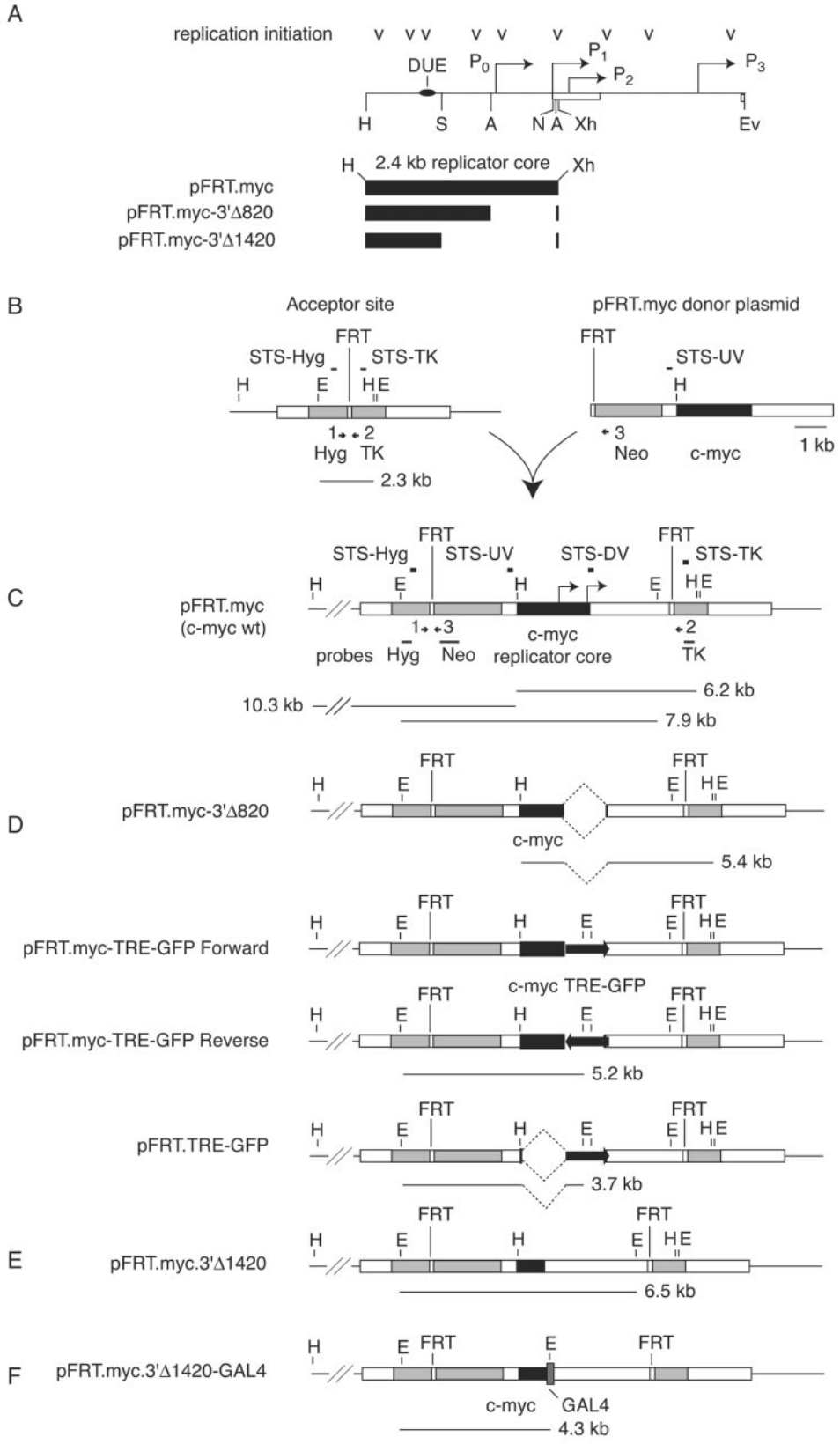
Generation of stable cell lines. A 2.4-kb HindIII-XhoI fragment upstream of the human c-*myc* gene contains multiple replication initiation sites and acts as a chromosomal replicator when transferred to an ectopic genomic target site (51, 52) (Fig. 1A). This fragment contains the c-*myc* P_0 and P_1 promoters, several transcription factor consensus binding sites, and an organized nucleosome array stable with respect to chromosomal translocation (20, 35, 37, 44, 45, 67, 71, 78, 87). To analyze the effect of transcription on replicator activity, clonal cell lines were constructed using the yeast FLP recombinase to integrate donor plasmids containing wild-type or mutated versions of the c-*myc* core replicator in the FRT site in the HeLa/406 acceptor cell line (Fig. 1B to F) (51, 52). For pFRT.myc .TRE-GFP cell lines, the c-*myc* promoter elements contained in the c-*myc* ApaI-ApaI fragment (nt 1539 to 2367) were replaced with a GFP transcription unit cloned in either orientation. The forward orientation is defined as the placement of the GFP gene in the same transcriptional sense as that of the endogenous c-*myc* gene downstream of the c-*myc* replicator sequences (Fig. 1D).

In pFRT.myc.TRE-GFP, the tetracycline-controlled transcriptional silencer tTS binds the *tetO* sequence of the tetracycline response element (TRE). In the presence of the doxycycline ligand, tTS is released and the reverse tetracycline repressor (rTetR-VP16) binds the TRE to activate transcription (26, 30). Additional cell lines were constructed containing inactive c-*myc* replicator deletion mutants pFRT.myc.3′Δ820, missing the Apa1-Apa1 c-*myc* DNA fragment (Fig. 1D), pFRT.myc.3′Δ1420, missing the c-*myc* Spe1-Not1 c-*myc* DNA fragment (Fig. 1E), and pFRT.myc.3′Δ1420-GAL4, in which the 1,420-bp Spe1-Not1 fragment was replaced by a 125-bp oligonucleotide containing four binding sites for the *S. cerevisiae* GAL4 protein (Fig. 1F).

Targeted integration was accomplished by FLP recombinase-mediated recombination between FRT sites in the c-*myc* donor plasmids and the acceptor site located at chromosome position 18p11.22 (nt 8,947,503; G. Liu, unpublished data) (Fig. 1B). The acceptor site FRT is positioned within the Hyg-TK gene without disrupting the reading frame of the fusion protein. FLP-mediated integration of the pFRT.myc donor plasmid or its derivatives displaces the TK part of the gene with a neomycin phosphotransferase coding sequence, rendering correctly targeted cells resistant to G418 and ganciclovir. Accurate integration of all constructs was confirmed by analytical PCR using primers that generated diagnostic products from the empty or occupied acceptor sites and by Southern blot hybridization using probes complementary to the acceptor site (Hyg probe, TK probe) or origin donor plasmid (Neo probe). As seen in Fig. 2, diagnostic primers 1 and 2 yielded amplified product only with HeLa/406 cell DNA containing the unoccupied acceptor site but not from cells with the acceptor site occupied by any of the donor plasmids (Fig. 2A). Conversely, primers 1 and 3 yielded the expected amplified product only with DNA from cells with an occupied acceptor site. Hybridization with the Hyg (Fig. 2B and E) or TK (Fig. 2D) probes confirmed that integration occurred at the intended chromosomal location, and hybridization with the Neo probe yielded a single hybridizing band, excluding the possibility that integration may also have taken place at a location other than the acceptor FRT site (Fig. 2B, C, and E). In cells containing integrated pFRT.TRE-GFP, targeting to the acceptor site was confirmed by single-copy hybridization of the Neo probe to an internal EcoRI fragment and to a single HindIII junction fragment spanning the acceptor and donor DNAs (Fig. 2C).

Analysis of gene expression. Clonal pFRT.myc.TRE-GFP cell lines were stably transfected to express both the rtTA transcriptional activator and the tTS transcriptional repressor, and derivative clonal lines were selected in which GFP expression was regulated by doxycycline. pFRT.TRE-GFP, an additional plasmid containing a doxycycline-inducible GFP gene, was used to construct a control cell line with the TRE-GFP construct from which all but the 5′ 79 bp of c-*myc* sequence had been removed.

Q-PCR of cDNAs from transiently transfected cells verified the inducible GFP expression from these plasmids. Thus, doxycycline increased expression from pFRT.myc.TRE-GFP or pFRT.TRE-GFP by ~6- to 10-fold or ~15-fold, respectively (Fig. 3A). In the stable cell lines, microscopic observation revealed GFP expression in the pFRT.myc.TRE-GFP (forward) cell line 24 h after doxycycline induction (Fig. 3B) and in the pFRT.TRE-GFP line (data not shown) but not in the pFRT.myc.TRE-GFP reverse cell line. Northern blot analysis confirmed that GFP expression was significantly lower in the pFRT.myc.TRE-GFP reverse cell line than in the forward cell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replication initiation
DUE
P0
P1
P2
P3
H
S
A
N
A
Xh
Ev
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pFRT.myc-3'Δ820
pFRT.myc-3'Δ1420
B
Acceptor site
pFRT.myc donor plasmid
FRT
STS-Hyg
STS-TK
STS-UV
Hyg
TK
Neo
c-myc
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c-myc
replicator core
10.3 kb
6.2 kb
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D
pFRT.myc-3'Δ820
5.4 kb
pFRT.myc-TRE-GFP Forward
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pFRT.myc-TRE-GFP Reverse
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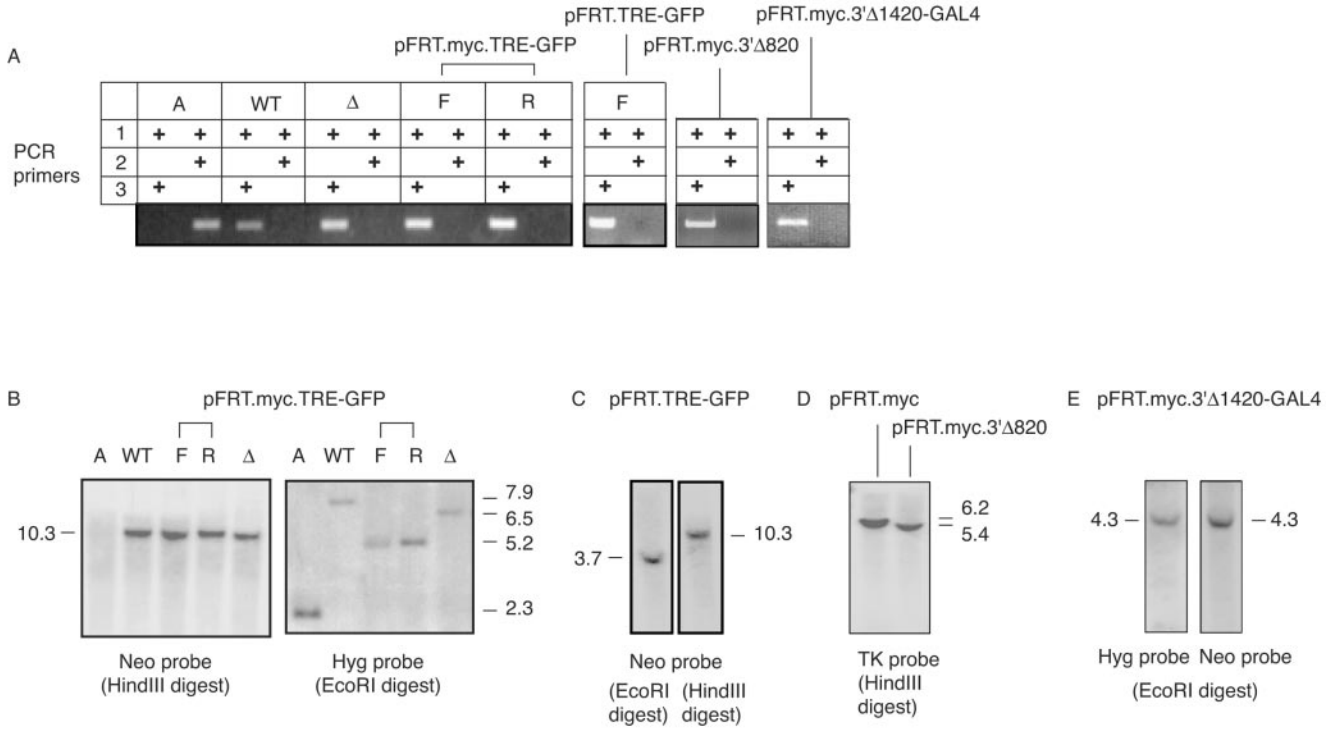

FIG. 2. Structural confirmation of ectopic c-*myc* cell lines. (A) Genomic DNA was isolated from the cell lines whose results are shown in Fig. 1 and amplified by PCR with primers yielding products diagnostic for the unoccupied acceptor site (primers 1 and 2) or the occupied acceptor site (primers 1 and 3). A, acceptor HeLa/406 cells; WT, Δ, F, and R, TRE-GFP cells in forward orientation (F), in reverse orientation (R), with pFRT.myc integrated (WT), and with pFRT.myc.3′Δ1420 integrated (Δ). (B) Hybridization to DNA from acceptor cells or cells transfected with pFRT constructs. Left, hybridization of the Neo probe internal to the pFRT donor plasmids to HindIII junction fragments between the acceptor site and chromosomal DNA. Right, hybridization of the acceptor site Hyg probe to EcoRI junction fragments. (C) Hybridization of the Neo probe to EcoRI-digested junction fragments (left) or HindIII junction fragments (right) from cells with integrated with pFRT.TRE-GFP. (D) Hybridization of the TK probe to HindIII-digested junction fragments from cells with integrated pFRT.myc or pFRT.myc.3′Δ820. (E) Hybridization of the Hyg probe (left) or the Neo probe (right) to EcoRI junction fragments from cells with integrated pFRT.myc.3′Δ1420-GAL4. Size markers are in kilobase pairs. The pFRT.myc.3′Δ1420 integrant has previously been characterized (51).

line (Fig. 3C). Therefore, chromosomal integration of the reporter genes at the ectopic site slightly decreased the degree to which the forward-orientation reporter genes could be induced but significantly decreased the induction of the reverse orientation gene. The orientation dependence of transgene expression has been observed previously and correlated with differences in DNA methylation (24, 48). Experiments using methylation-sensitive restriction enzymes did not indicate that the integrated genes become methylated (G. Randall, unpublished data); however, we have not pursued the basis for the orientation dependence of induced expression further.

To quantitate the induction of GFP expression in each of the

FIG. 1. Maps of DNA sequences used in this study. (A) HindIII-EcoRV fragment of the human c-*myc* locus. The first c-*myc* exon and the 5′ part of the second exon are indicated by open boxes. The HindIII-XhoI fragment contains the wild-type c-*myc* replicator core. (A, ApaI; Ev, EcoRv; H, HindIII; S, SpeI; N, NotI; Xh, XhoI). Downward-pointing arrowheads represent replication initiation sites previously mapped in vivo (6, 44, 45, 67, 78). The in vivo DNA unwinding element (DUE) (7, 23, 58) is indicated. P_0, P_1, P_2, P_3, c-*myc* promoters. A TATA box is located 28 bp 5′ to the P_1 promoter. (B) Left, the clonal acceptor cell line HeLa/406 was generated through single-copy insertion of the plasmid pHyg.FRT.TK, containing an FLP recombinase target site (FRT), into the HeLa genome. Hyg, hygromycin resistance gene; TK, herpes simplex virus thymidine kinase gene (shaded rectangles). STS, sequence-tagged sites (Q-PCR primers); numbered arrows, analytical PCR primers (cf. Fig. 2). Unfilled rectangles are vector sequences. The acceptor cell line is hygromycin resistant and ganciclovir sensitive. Right, the donor plasmid pFRT.myc containing a promoterless neomycin resistance gene (Neo) and the 2.4-kb HindIII-XhoI fragment of the c-*myc* replicator (solid black box) was integrated at the acceptor site by the FLP recombinase expressed from pOG44. (C) The resulting pFRT.myc cell line is resistant to hygromycin, neomycin, and ganciclovir. PCR primers 1, 2, and 3 (horizontal arrowheads) gave diagnostic PCR products for the empty acceptor site (primers 1 and 2) or after FLP-mediated integration of the donor plasmids (primers 1 and 3). Sequence-tagged sites for Q-PCR are indicated as STS-Hyg, STS-UV, STS-DV, and STS-TK. The positions of restriction sites (E, EcoRI; H, HindIII) and probes relevant to the Southern analysis are shown. Bars below each map correspond to diagnostic restriction fragments detected by Southern blotting. (D) The pFRT.myc.TET-GFP cell lines were generated by deleting the 3′ ApaI-ApaI c-*myc* sequence (pFRT.myc.3′Δ820, dashed lines) and replacing it with a GFP transcription unit under control of the tetracycline-inducible TRE promoter. The GFP genes were cloned in both orientations (indicated by thick arrows) relative to the c-*myc* sequences. All c-*myc* sequences except the 5′ HindIII-PinI fragment (79 bp) had been deleted from the pFRT.TRE-GFP cells. (E) The 3′ 1,420 bp of the c-*myc* core origin sequence was deleted from the donor plasmid pFRT.myc to construct the cell line pFRT.myc.3′Δ1420. (F) pFRT.myc.3′Δ1420-GAL4 was constructed by fusing four GAL4p binding sites to the 3′ end of the c-*myc* sequences in pFRT.myc.3′Δ1420.

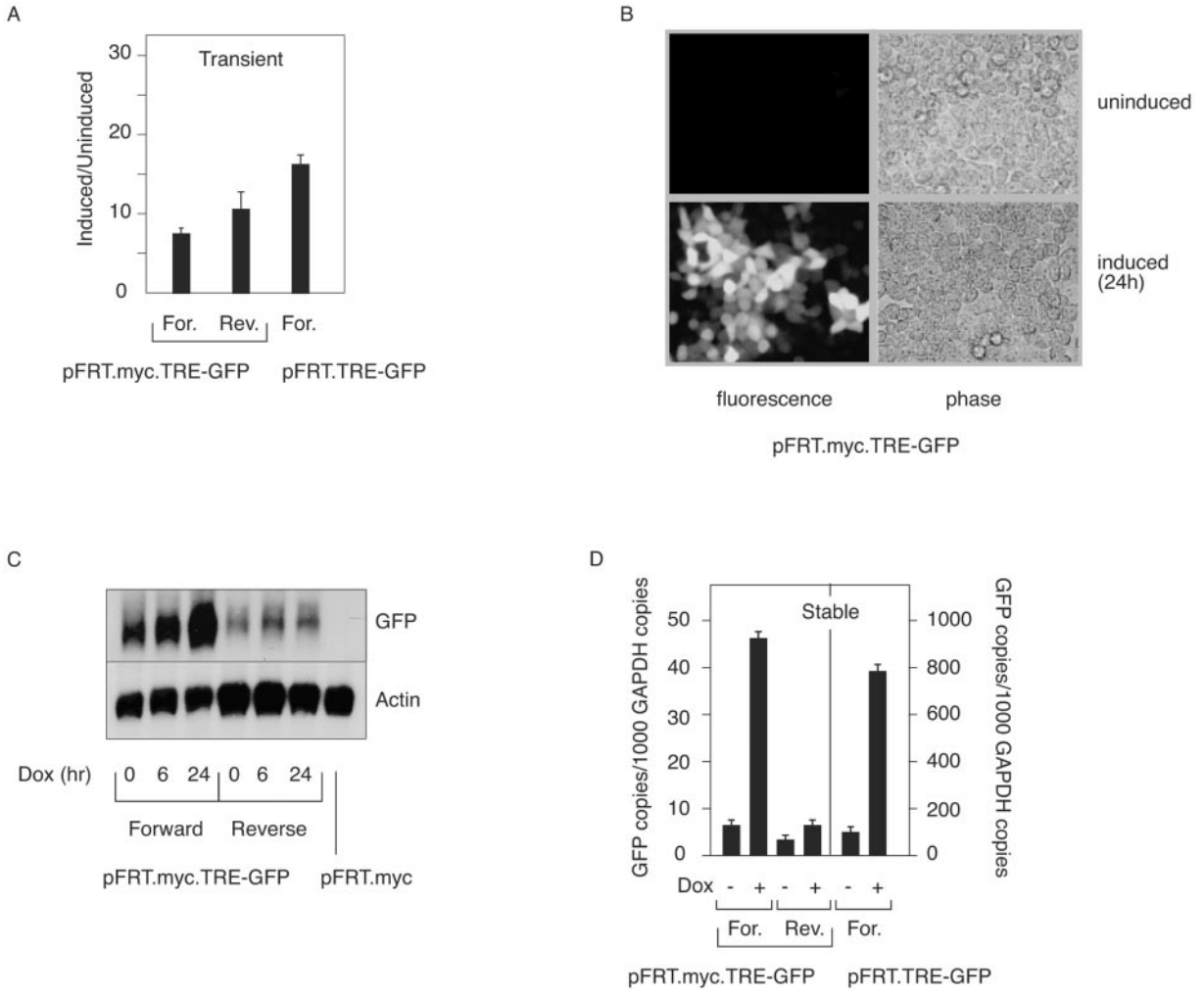

FIG. 3. Induction of GFP gene expression. (A) GFP expression from transiently transfected plasmids. Q-PCR was carried out with cDNA reverse transcribed from total RNA from cells transiently transfected with pFRT.myc.TET-GFP or pFRT.TRE-GFP and treated with doxycycline (6 h) or left untreated. The data are presented as the ratio of signals from sister cell cultures with and without ligand treatment, normalized for cotransfected β-galactosidase plasmid expression. Error bars indicate standard deviations. For., forward; Rev., reverse. (B) Effect of doxycycline treatment (24 h) on GFP expression in cells containing stably integrated pFRT.myc.TRE-GFP (forward orientation). (C) Time course Northern analysis of pFRT.myc.TRE-GFP expression in cell lines upon doxycycline (Dox) induction, hybridized successively with probes for GFP and β-actin. RNA from cells containing stably integrated pFRT.myc (c-*myc* wild type) was used as a negative control. (D) Q-PCR was carried out with cDNA reverse transcribed from total RNA from cells stably transfected with the pFRT.myc.TET-GFP or pFRT.TRE-GFP plasmids and treated with doxycycline (6 h) or left untreated. The data represent copies of GFP cDNA per 1,000 copies of GAPDH cDNA. Error bars indicate standard deviations.

stable GFP cell lines, real-time PCR was carried out on reverse-transcribed cDNA by use of primers complementary to GFP or an internal control, GAPDH cDNA. Relative to the expression of GAPDH, doxycycline strongly (~7-fold) induced GFP expression in the pFRT.TRE-GFP and pFRT.myc.TRE-GFP (forward) cell lines and modestly (~2-fold) induced expression in the pFRT.myc.TRE-GFP (reverse) cell line (Fig. 3D).

To determine whether structural changes in the chromatin of the integrated pFRT.myc.TRE-GFP constructs were correlated with their levels of gene expression, micrococcal nuclease (MNase) was used to digest nuclei from these cell lines. As shown in Fig. 4A, although local changes in the structure of the TRE were detectable on the original autoradiograms after induction, no large-scale differences were evident between the nuclease digestion patterns of the integrated plasmids containing uninduced versus induced genes. At the sensitivity of this assay, these data suggest that gross structural changes are not seen encompassing the c-*myc* replicator sequences between the cell lines irrespective of the induction of gene expression from the transcription units.

As a further assay of chromatin structure, ChIP was performed using anti-acetyl histone H4 antibody, and DNA sequences across the ectopic c-*myc* locus were quantitated in the immunoprecipitates (Fig. 4B). In the uninduced state all sites assayed showed hyperacetylation of about 2- to 10-fold relative to that seen with a sequence-tagged site in the transcriptionally inactive β-globin locus, with the highest levels of acetyl-H4 in the region of the constitutively expressed Hyg gene. Enhanced hyperacetylation of the ectopic locus was observed upon in-

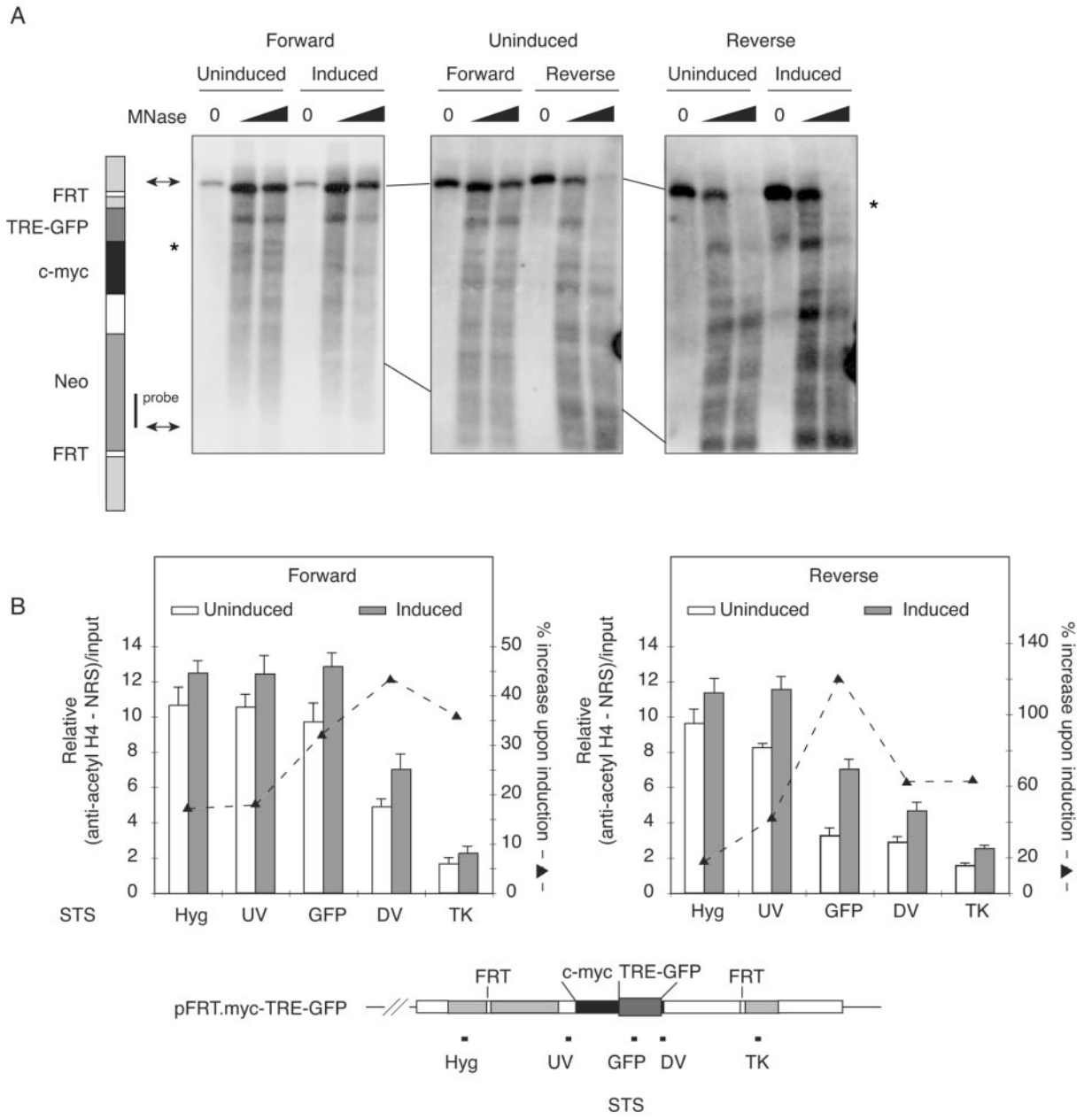

FIG. 4. Analysis of chromatin structure. (A) Nuclei were isolated from the pFRT.myc.TRE-GFP forward and reverse cell lines with or without doxycycline induction and subjected to increasing amount of micrococcal nuclease digestion (solid triangles). The DNA was purified, digested with NcoI (arrowheads), and hybridized to the Neo probe (solid bar). Asterisks indicate the TRE promoter regions of the forward and reverse TRE-GFP constructs. (B) Formaldehyde cross-linked chromatin was isolated from uninduced cells or doxycycline-treated cells (6 h) and immunoprecipitated with anti-acetyl histone H4 antibody or normal rabbit serum (NRS). Immunoprecipitated DNA was quantitated at the indicated STSs (Hyg, UV, GFP, DV, and TK) and at STS-54.8 in the human β-globin locus by Q-PCR. The data on the left-hand *y* axes are expressed relative to STS-54.8. The data on the right-hand *y* axes show percent increase in H4 acetylation calculated as follows: (100% × uninduced/induced) − 100%. Error bars indicate standard deviations.

duction of GFP expression, with the greatest increases in H4 acetylation observed over the GFP gene and its downstream flanking DNA. Moreover, the level of H4 acetylation of the forward-orientation GFP gene was 2 to 3 times that of the reverse orientation GFP gene, irrespective of induction. Thus, the level of H4 acetylation qualitatively, but not quantitatively, mirrored the expression levels of these genes. Overall, the level of histone acetylation, the degree and pattern of MNase sensitivity, and the profile of decreasing histone H4 acetylation from upstream to downstream across the ectopic locus did not show dramatic changes upon induction of transcription. We conclude that the induction of transcription is not associated with further large-scale changes in chromatin structure at the already accessible ectopic site.

Quantitation of nascent strand abundance. As a measure of replication origin activity, the abundance of short (1- to 2-kb) nascent DNA strands in asynchronous populations of clonally expanded cells containing pFRT.myc.TRE-GFP was determined at the ectopic c-*myc* replicator sites before and after induction of transcription. Nascent DNA was isolated by cell lysis and electrophoresis in alkaline gels (51, 52). This method eliminates all handling of DNA prior to size fractionation and avoids potential variability in nascent DNA isolation results. As has been reported previously, normalization of the nascent strand abundance at the ectopic site to the abundance at an internal reference site minimizes the standard deviation of these measurements (51, 52, 85). The internal reference for these studies was the β-globin STS-54.8 (41). Quantitative real-time PCR (Q-PCR) was used to compare the origin activity of the uninduced and induced pFRT.myc.TRE-GFP constructs with the activity of the wild-type pFRT.myc and the inactive pFRT.myc.3′Δ1420 constructs.

As shown in Fig. 5, in the absence of doxycycline induction, the presence of the TRE-GFP gene in either orientation elevated origin activity at STS-UV approximately 10- to 15-fold compared to the results seen with the inactive c-*myc* deletion construct pFRT.myc.3′Δ1420 or pFRT.myc.3′Δ820 and to levels roughly twice those of the wild-type replicator (pFRT.myc), with only minor changes at STS-Hyg or STS-TK. That the uninduced GFP transcription unit can substitute functionally for the endogenous c-*myc* promoter region suggests that some aspect of basal transcription strongly stimulates origin activity. On the other hand, induction with doxycycline decreased the abundance of short nascent strands at STS-UV in both the forward and reverse cell lines to approximately the level of that in the pFRT.myc cell line. Similar results were also obtained using competitive PCR to quantitate nascent strand abundances before and after induction of transcription (data not shown). Thus, the induction of transcription suppressed but did not eliminate replicator function. As summarized in Table 2, there was not the same quantitative relationship between the extent of induced transcription and the decrease in nascent strand abundance in the forward and reverse pFRT.myc.TRE-GFP cell lines. Both the forward and reverse cell lines showed a decrease of approximately fourfold in nascent DNA levels at STS-UV despite a sevenfold induction of transcription in the forward cell line and a twofold induction in the reverse cell line. One possible explanation for these observations is that the replicator is sensitive to the duration of transcription complex occupancy on the GFP gene. Experiments performed using α-amanitin to test this possibility are described below.

To confirm that the TRE-GFP transcription unit contributed to restoring c-*myc* replicator activity, an additional cell line was constructed containing pFRT.myc.3′Δ820, which is the vector for the inducible GFP constructs. Q-PCR was used to compare the levels of abundance of nascent strands at STS-Hyg, STS-UV, and STS-TK in cell lines containing the wild-type c-*myc* replicator pFRT.myc, a mutant c-*myc* replicator (pFRT.myc.3′Δ1420) shown to be as inactive as the empty acceptor site or a negative genomic control construct (51, 52), and the pFRT.myc.3′Δ820 mutant. Earlier results had shown that deletion of 200-bp segments from the 3′ region of the replicator (nt 1533 to 1932 or nt 2134 to 2337), reduced or eliminated origin activity (51). As expected from these prior results, the pFRT.myc.3′Δ820 construct was inert in the nascent-strand-abundance assay (Fig. 5C).

TABLE 2. Expression and replication in GFP cell lines

Construct	Expression[a]	Replication[b]
pFRT.myc	NA[c]	5.5 (1.0)
pFRT.TRE-GFP, forward, uninduced	84 (19)	1 (0.4)
pFRT.TRE-GFP, forward, induced	783 (25)	0.8 (0.6)
pFRT.myc.TRE-GFP, forward, uninduced	6 (1)	14.5 (1.5)
pFRT.myc.TRE-GFP, forward, induced	46 (2)	3.5 (1.0)
pFRT.myc.TRE-GFP, forward, uninduced, + α	NA	4 (1.0)
pFRT.myc.TRE-GFP, forward, induced, + α	NA	6.5 (1.5)
pFRT.myc.TRE-GFP, reverse, uninduced	3 (1)	19 (1.0)
pFRT.myc.TRE-GFP, reverse, induced	7 (1)	5.5 (0.8)
pFRT.myc.TRE-GFP, reverse, uninduced, + α	NA	13.5 (1.0)
pFRT.myc.TRE-GFP, reverse, induced, + α	NA	13.0 (1.0)

[a] GFP mRNA was measured by reverse transcription real-time PCR and is expressed as copies (standard deviation) per 1,000 copies of GAPDH. Data are from Figure 3.

[b] Nascent strand abundance (standard deviation) at STS-UV was measured as described in Materials and Methods and is expressed for each construct relative to the nascent strand abundance at STS-54.8 of the human β-globin locus (1, 2). "α" denotes α-amanitin treatment. Data are from Figure 5.

[c] NA, not assayed.

The replicator activity of the pFRT.myc.TRE-GFP constructs was also dependent on the presence of the c-*myc* replicator sequences, since pFRT.TRE-GFP, from which all but the 5′ 79 bp of c-*myc* sequence were removed, did not show replicator activity when integrated at the same chromosomal location (Fig. 5D and Table 2). Because the basal level of expression from pFRT.TRE-GFP was much higher than that from pFRT.myc.TRE-GFP (Fig. 3), it was possible that the presence of the c-*myc* sequences stimulated replication activity not because of a positive replicator function but because they negatively affected the basal level of GFP expression. This does not appear to be the case, however, since replicator activity was not seen at the reverse-orientation gene whose induced expression was comparable to that of the basal expression of the forward gene, and GFP expression at the same level as that of pFRT.myc.TRE-GFP, but in analogous ecdysone-dependent GFP promoter construct cell lines, did not enable replication activity of the GFP transcription unit (data not shown).

α-Amanitin treatment. Irrespective of polarity, both forward and reverse pFRT.myc.TRE-GFP cell lines showed greater origin activity when uninduced and a decrease in origin activity upon transcriptional induction. To analyze this effect further we used α-amanitin, which inhibits transcription in HeLa cells by strongly blocking the progress of engaged RNA Pol II molecules but does not displace the enzyme from its template (3, 42, 49, 59, 72). When cells were treated with α-amanitin a polar effect of gene orientation was observed. In uninduced cells harboring the forward orientation of pFRT.myc.TRE-GFP, α-amanitin reduced the origin activity at STS-UV approximately threefold to levels similar to those of induced cells, whereas in the reverse orientation, α-amanitin had little effect on nascent strand abundance in uninduced cells (Fig. 5 and Table 2). Cells were also treated with α-amanitin following induction of transcription with doxycycline. As shown in Fig. 5, for both the forward and reverse pFRT.myc.TRE-GFP cell lines the effects of α-amanitin on nascent strand abundance were highly similar whether transcription had been induced with doxycycline or not.

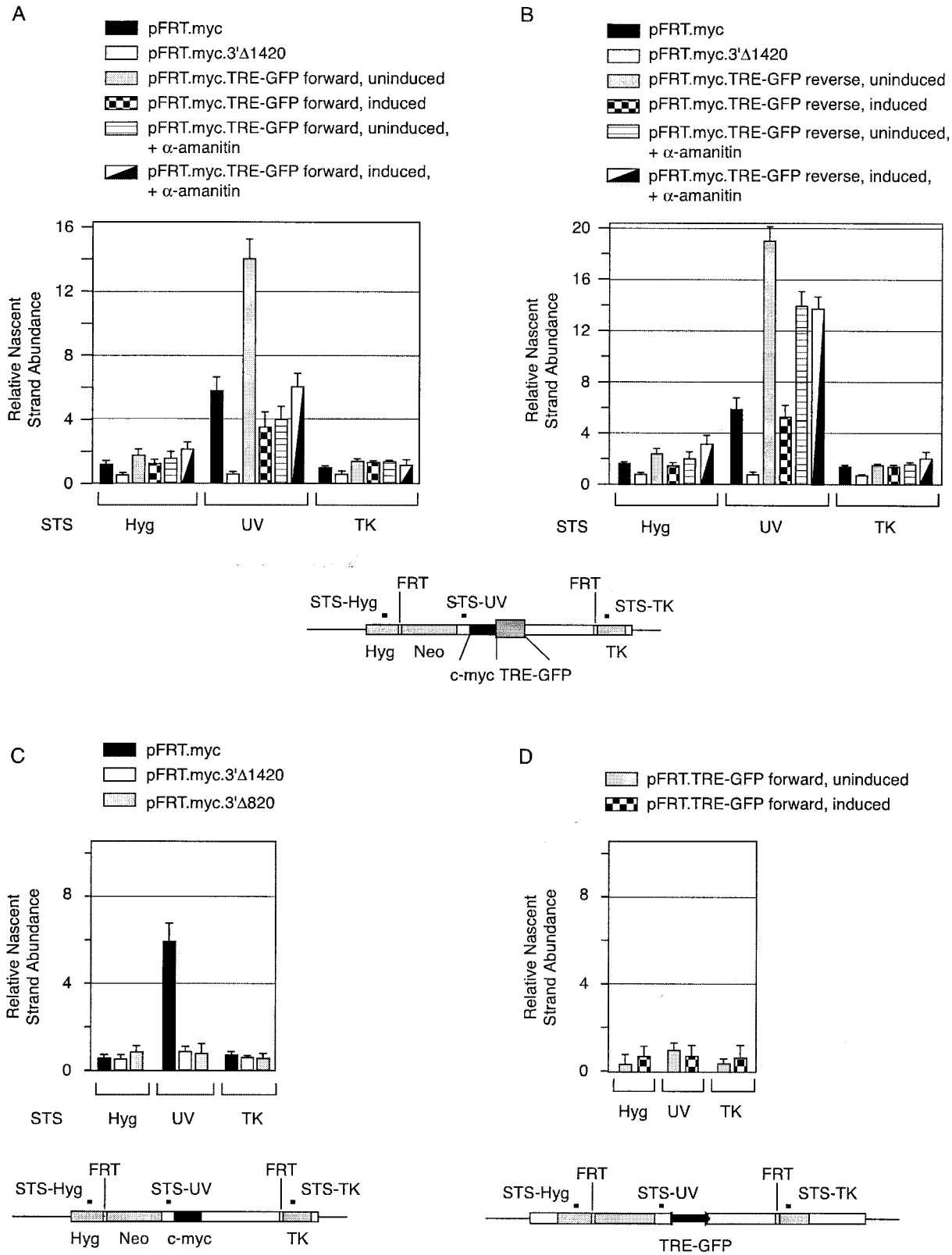

FIG. 5. Nascent strand abundance and Q-PCR analysis. (A and B) The abundance of short nascent strands at STS-Hyg, STS-UV, and STS-TK in cells containing pFRT.myc.TRE-GFP in the forward orientation (A) or the reverse orientation (B) was analyzed by real-time PCR. Cells were left uninduced, treated with doxycycline (6 h), or treated with α-amanitin (1 h) in the presence or absence of inducer. The nascent strand abundance in cells containing integrated pFRT.myc or pFRT.myc.3′Δ1420 is shown for comparison. Values shown are relative to nascent strand abundance at β-globin STS-54.8 (41). (C) Absence of replicator activity in control constructs. The abundance of short nascent strands at the indicated STSs was measured in cell lines containing pFRT.myc (wild-type core replicator) or the deletion mutant pFRT.myc.3′Δ1420 or pFRT.myc.3′Δ820. (D) Absence of replicator activity in cells containing pFRT.TRE-GFP under induced or uninduced conditions. Values shown are relative to nascent strand abundance at β-globin STS-54.8. Error bars indicate standard deviations.

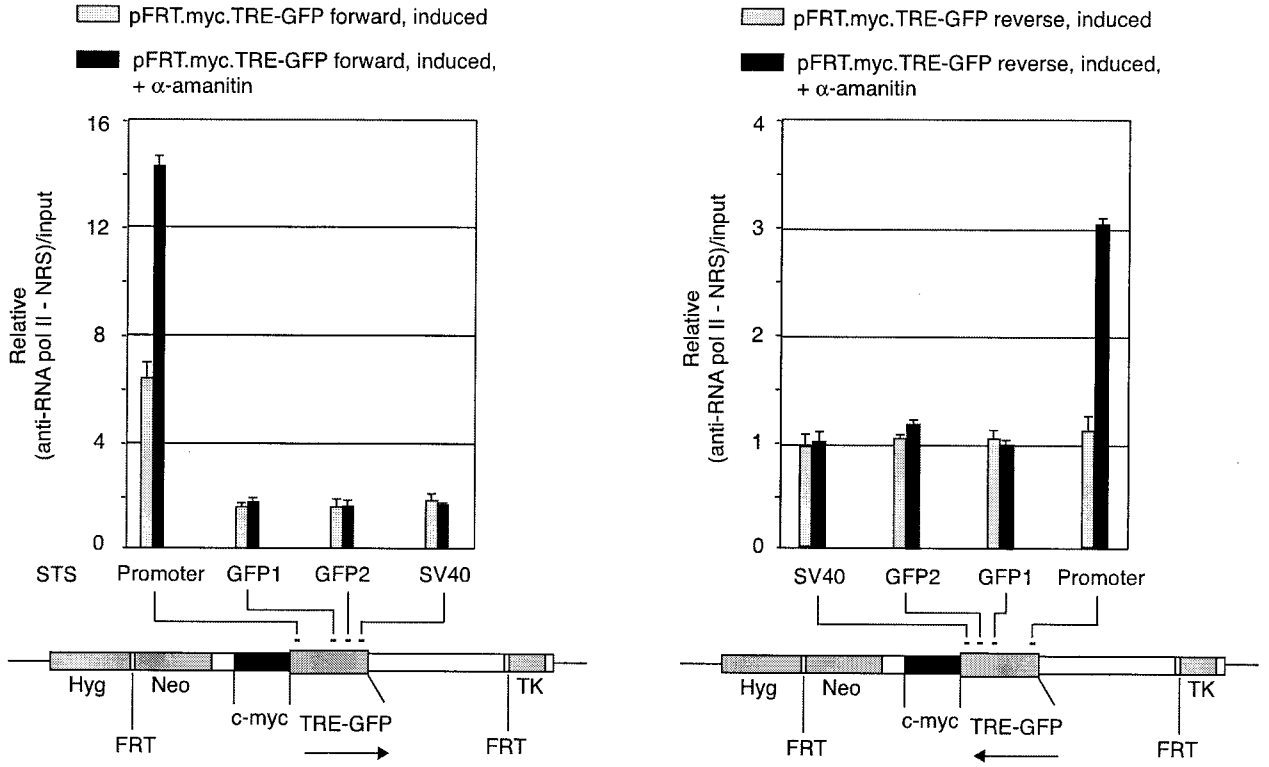

FIG. 6. RNA Pol II ChIP. Formaldehyde cross-linked chromatin was isolated from forward and reverse orientation cell lines that were left uninduced, treated with doxycycline (6 h), or treated with doxycycline (6 h) and α-amanitin (1 h). Chromatin was immunoprecipitated with antibodies against RNA Pol II or normal rabbit serum. Q-PCR of the immunoprecipitated DNA was carried out at the STSs indicated. The data are represented as the abundance of Pol II-bound DNA in the induced or induced plus α-amanitin-treated cells relative to that of the RNA Pol II bound DNA in uninduced cells. Error bars indicate standard deviations.

These results implied that compared to the results seen with the uninduced state, increased passage of the RNA Pol II transcription complex across the TRE-GFP gene reduced origin activity, as did stalling of the transcription complex proximal to the replicator, but that stalling of the transcription complex distal to the replicator was more permissive of origin activity. ChIP was performed to confirm that RNA Pol II was bound at the expected sites under these experimental conditions. As observed in similar experiments (63, 74, 77), the greatest abundance of Pol II-bound sequences was near the promoter (Fig. 6). Mirroring the induction of GFP expression observed by Northern blotting or reverse transcription PCR, doxycycline increased the amount of promoter sequences bound by Pol II approximately sixfold in the pFRT.myc.TRE-GFP forward-orientation cell line whereas no significant change was observed in the reverse orientation cell line. α-Amanitin treatment during the last hour of doxycycline induction further increased the binding of RNA Pol II at the promoter region of the GFP genes two- to threefold. However, the dramatic inhibition of replication in the presence of doxycycline and α-amanitin was only observed in the forward-orientation cell line (cf. Fig. 5). Taken together, these results suggest that the increased association of the Pol II complex with the TRE-GFP genes or stalling of the Pol II complex near the replicator adversely affected replication initiation.

CREB-GAL4p expression restores origin activity to an inactive replicator. To assess directly whether transcription factor binding per se could stimulate replication, the cell line pFRT.myc.3′Δ1420-GAL4 was constructed in which the promoter sequences missing from pFRT.myc.3′Δ1420 were replaced with a GAL4p binding cassette (Fig. 1F). The GAL4p DNA binding domain or a fusion protein comprising the amino terminal transcription activation domain of the cyclic AMP response element binding protein CREB and the DNA binding domain of GAL4p (69) were expressed at similar levels in these cells or in pFRT.myc.3′Δ1420 as a control (Fig. 7A), and replication initiation activity was measured. Expression of CREB-GAL4p rescued replication initiation activity in the pFRT.myc.3′Δ1420-GAL4 cells but not in pFRT.myc.3′Δ1420 cells lacking the GAL4p binding site (Fig. 7B). In contrast, GAL4p expression could not rescue replication activity in the pFRT.myc.3′Δ1420-GAL4 cells. Expression of CREB-GAL4p or GAL4p did not affect replication activity at the endogenous c-*myc* replicator (Fig. 7B) or at the β-globin locus (data not shown); thus, the effect of CREB-GAL4p expression was limited to the region near the GAL4p binding site. ChIP analysis (Fig. 7C) indicated that the expression of CREB-GAL4p correlated with a local increase in histone H4 acetylation near the GAL4p binding site. These results indicate that the GAL4 target cassette is accessible for binding, that CREB-GAL4p is able to recruit histone acetyltransferase activity to this site, and that targeted transcription factor binding can rescue the activity of the inactive c-*myc* replicator.

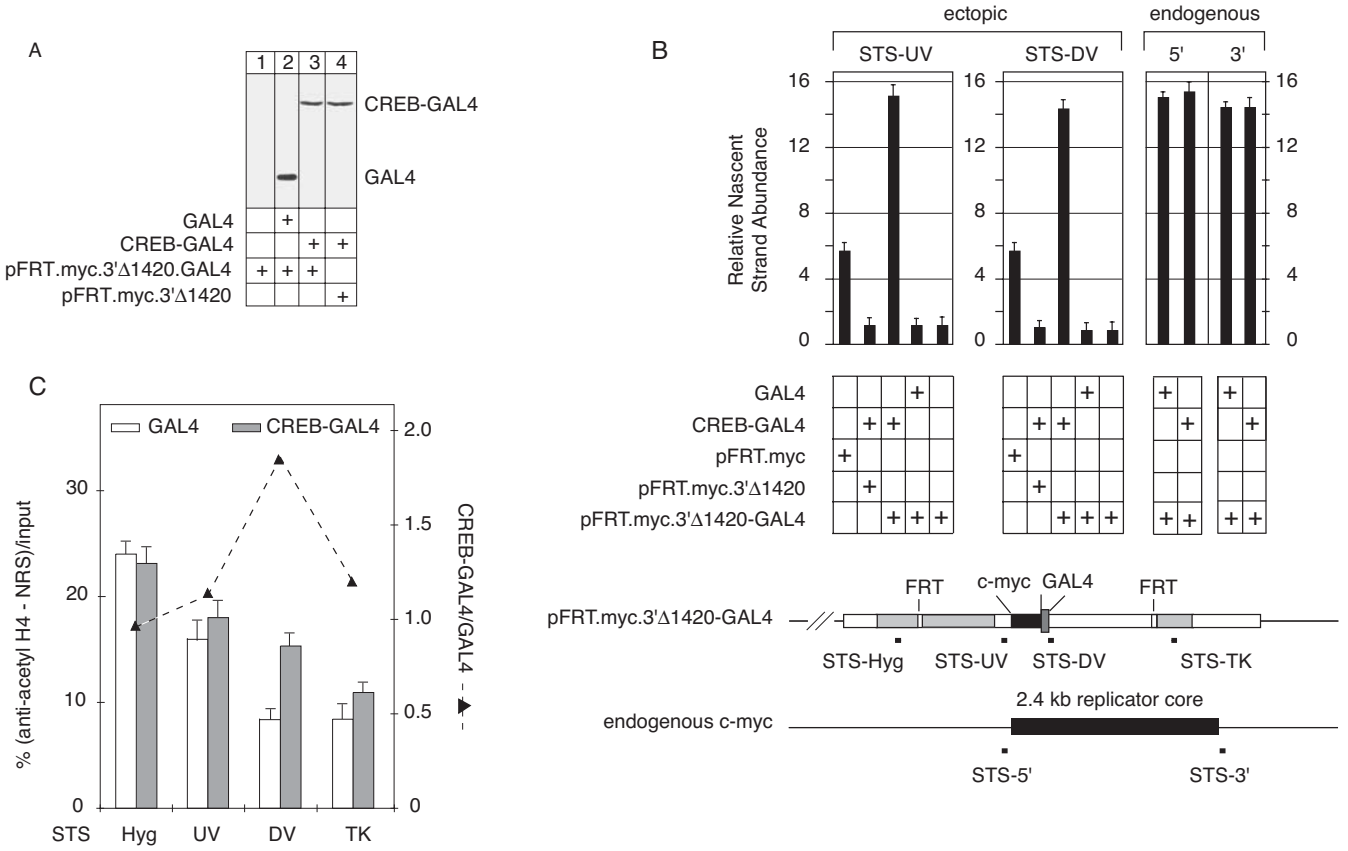

FIG. 7. Expression of CREB-GAL4p restores origin activity to pFRT.myc.3′Δ1420-GAL4. (A) GAL4p or CREB-GAL4p was transiently expressed in the indicated cell lines. Western blot, anti-GAL4p antibody. Equal amounts of cell extract (actin control not shown) were loaded. (B) Nascent strand abundance was measured at STS-UV and STS-DV in the indicated cell lines with or without GAL4p or CREB-GAL4p expression (48 h posttransfection). Nascent strand abundances were normalized for CREB-GAL4 and GAL4 plasmid transfection efficiency (50 to 54%) as determined by flow cytometry (Materials and Methods). For comparison, the levels of nascent strands 5′ and 3′ to the endogenous c-*myc* 2.4-kb core replicator were measured. Values shown are relative to nascent strand abundance at β-globin STS-54.8. Error bars indicate standard deviations. (C) Formaldehyde cross-linked chromatin was immunoprecipitated from pFRT.myc.3′Δ1420-GAL4 cells transfected with GAL4p- or CREB-GAL4p-expressing plasmids, and the indicated STSs were quantitated in the immunoprecipitated DNA by real-time PCR. Error bars indicate standard deviations.

DISCUSSION

Replication and transcription begin at nonrandom sites along eukaryotic chromosomes, but despite the general early S-phase replication of transcribed sequences, attempts to compare levels of origin activity proximal to promoters in cells where the promoter is active versus inactive promoter results have produced both positive (46, 81) and negative (40, 60) correlations. Thus, the observed differences in origin activity may derive from a property of each cell type other than active transcription. When autonomously replicating plasmids harboring a constitutively expressed selectable marker gene and an inducible reporter gene were assayed in human 293S cells, expression of the marker gene was compatible with replication but induced transcription of the reporter gene was not (31).

Here we extend these observations to a chromosomal context. Previous work has shown that the chromosomally integrated c-*myc* replicator construct pFRT.myc.3′Δ1420, which is missing the 3′ portion of the replicator, and mutants from which individual ~200-bp blocks containing transcription factor binding sites had been deleted do not show origin activity (51). To test the hypothesis that the presence of a transcription unit facilitates replication initiation, clonal cell lines were constructed containing single-copy chimeric replicators in which the c-*myc* 3′ transcription factor binding sites (9, 20, 35, 37, 67, 71, 78, 87) were replaced by inducible transcription units. The origin activity of all constructs was monitored after integration at the same chromosomal location. The transcription units were placed in both orientations relative to the replicator sequences to assay for polar effects in constructs where replication and transcription forks were moving in the same or opposite directions.

The replicator-GFP fusion plasmids showed significant induction of GFP gene expression in transient assays. In contrast, whereas single-copy integrants of pFRT.myc.TRE-GFP in the forward orientation showed induction quantitatively similar to that seen with transiently transfected cultures, the presence of doxycycline only modestly induced expression of the integrated pFRT.myc.TRE-GFP reverse construct. It is conceivable that these differences stem from comparing expression of a population of transiently transfected plasmid to that from a clonal

cohort of a single integrated plasmid. However, we do not believe these effects on transcription are due to mutation of the integrated plasmids undetected by Southern blotting or PCR, since doxycycline induction of the forward and reverse pFRT .myc.TRE-GFP integrants had the same effect on replication.

As reported previously, the greatest abundance of short nascent DNAs was detected closest to the c-*myc* replicator sequences (51). Prior to induction the pFRT.myc.TRE-GFP constructs showed >15-fold-elevated origin activity at STS-UV compared to the results seen with mutant pFRT.myc.3′Δ1420. This observation suggests that replacement of the endogenous c-*myc* transcription factor binding site region with a heterologous transcription factor-binding region can restore replicator activity. Since integration of an inducible TRE-GFP gene without the bulk of c-*myc* replicator sequences at the same chromosomal location did not engender replicator activity, this effect appears to be dependent on cooperation between the c-*myc* sequences and the transcription unit. A background level of expression is detected from each of the transcription units in the absence of induction. Among other possibilities, the chromatin structure correlated with the basal expression of the TRE-GFP transcription units may also be conducive to site-specific replication initiation. Alternatively, since we have not mapped the sites of transcription initiation in the uninduced versus induced states, it is formally possible that the transcription start sites for background expression are compatible with replication while the start sites for induced expression are not. However, the results of Pol II ChIP and the orientation-dependent effects on replication of inhibiting transcription with α-amanitin argue against this, as does the observation that CREB-GAL4p binding in the absence of known promoter sequences restores replication activity.

Binding of the CREB-GAL4p transcription factor to the inactive pFRT.myc.3′Δ1420-GAL4 replicator was associated with restoration of origin activity. Although we have not constructed a pFRT.GAL4 cell line devoid of c-*myc* replicator sequences, earlier data from other laboratories (48, 65) as well as from our own (51), and the data presented in this report, show that transcription factor binding sites are insufficient for origin activity in the absence of replicator elements. We believe, therefore, that the binding of CREB-GAL4p acts in concert with sequence elements (58) or proteins (23, 33) associated with the 5′ 1,000 bp of the c-*myc* replicator. Work to be presented elsewhere in which we describe the identification in a yeast one-hybrid screen of a previously uncharacterized protein that binds to the c-*myc* DUE in vivo and modulates replication complex assembly in vitro is potentially relevant.

While the present work was in progress it was reported that transgenes containing transcription factor binding sites from the human β-globin locus control region (hypersensitive sites 2, 3, and 4; LCR HS2-4) (39, 62, 79) fused upstream of a β-globin promoter and EGFP gene were targeted into the mouse genome (24, 48). In two of three chromosomal locations the transgenes showed orientation-dependent expression correlated with changes in DNA methylation. In one of these cell lines, the transcriptionally permissive orientation was associated with local histone H4 hyperacetylation and early S-phase activation of the otherwise late-replicating insertion site. This effect was retained in a construct missing the β-globin promoter but containing the LCR HS2-4 (48). In comparison, a recent study of the nonexpressed but decondensed chicken β-globin locus did not find a consistent correlation between origin activity and histone acetylation (68). These observations are consistent with the conclusion that transcription factor binding and histone acetylation may be two of several aspects of chromatin structure that can promote origin activity.

When expression from pFRT.myc.TRE-GFP was induced by doxycycline, origin activity was decreased approximately to the levels observed from the wild-type c-*myc* replicator at the ectopic locus but not to levels as low as those seen with the pFRT.myc.3′Δ1420 mutant. These data imply that the process of induced transcription inhibits replication. The similarity of nuclease digestion patterns over the uninduced and induced loci suggests that the inhibition of replication occurs without stable, large-scale movement of nucleosomes, although the structure of chromatin is modified by histone hyperacetylation. One interpretation of the absence of a polar effect of transcription is that ample positive or negative torsional strain does not accumulate to affect replication differentially. Alternatively, replication may be inhibited by states repressive for replication at both the 5′ and 3′ ends of the transcription unit.

α-Amanitin binds RNA Pol II (42, 49, 72) and dramatically slows transcription in HeLa cells (14). Treatment of the uninduced cell lines to block background GFP gene expression did not result in a detectable change in the flow cytometry profile of the cultures (data not shown). Nascent DNA quantitation of α-amanitin-treated forward-orientation pFRT.myc.TRE-GFP cells decreased origin activity at STS-UV to levels comparable to those of induced cells, while α-amanitin treatment of reverse-orientation cells only marginally reduced origin activity. α-Amanitin neither dissociates the RNA Pol II-DNA-RNA complex nor competes for nucleotide incorporation but inhibits RNA Pol II translocation from several thousand to only a few bases per minute (15, 17, 18, 42, 72). That α-amanitin inhibition of basal and of induced transcription had similar effects on replication suggests that the identity of the transcription factor present at the promoter is less crucial than the residence of the transcription complex. Thus, in the presence of α-amanitin, a state inhibitory for replication may persist near the 5′ end of a transcription unit, while the inhibitory state at the 3′ end of the transcription unit is dissipated. Under these conditions, replication was inhibited in the forward-orientation cell line where the TRE-GFP promoter is proximal to the c-*myc* replicator sequences but was not inhibited in the reverse-orientation cell line.

Findings with the inducible cell lines revealed that a transcription unit restores origin activity at an inactive replicator while induced transcription represses origin activity irrespective of the orientation of the transcription unit relative to that of the replicator. The observation that transcriptional induction of the forward and reverse TRE-GFP constructs shows similar effects on replication but significantly different quantitative effects on transcription suggests that the structures of the promoter and replicator are qualitatively different under uninduced and induced conditions. Moreover, α-amanitin treatment mimics the effect of elevated transcription when the TRE promoter is proximal to the replicator, but the effect of the presence of α-amanitin is diminished when the TRE promoter is distal to the replicator. One hypothesis to incorporate these observations is that transcription factor binding enhances ori-

gin activity at the endogenous or ectopic c-*myc* replicators but that a later step in transcriptional activation is repressive to replication initiation. In similarity to a model proposed for chromatin structural changes by human HSF1-dependent transcription at the hsp70 promoter (14), elevated expression may interfere with origin activity by the establishment of a repressive chromatin structure for replication initiation near the promoter that is stable with respect to the presence of α-amanitin and by the α-amanitin-sensitive propagation of a repressive state with the transcription fork. Hence, in the presence of α-amanitin the relative locations of the promoter and replicator could account for the observed orientation-dependent effects on replication. In the context of the present results, binding of a transcriptional activator or coactivator near the c-*myc* replicator is conducive to replication initiation, while subsequent occupancy of a nearby promoter by the Pol II transcriptional machinery may inhibit origin activity.

The experiments presented here take advantage of a common genomic integration site to compare the effects of sequence deletions and substitutions and examine a single type of replicator. Thus, it is conceivable that these results are locus specific and that other replicators would use different strategies to regulate origin activity at other chromosomal sites. At the ectopic acceptor site the H4 histones are hyperacetylated, possibly due to the presence of the constitutively expressed Hyg^r gene or another aspect of chromatin structure. However, origin activity is not observed at the ectopic site in the absence of c-*myc* replicator sequences, and the origin activity of the wild-type 2.4-kb c-*myc* replicator at the ectopic location is similar to that at the endogenous c-*myc* locus on a per copy basis (Fig. 7). By contrast, the origin activity of pFRT .myc.3′Δ1420-GAL4 bound by CREB-GAL4p exceeds that of the endogenous c-*myc* locus. Since all of these constructs were assayed in the same chromosomal location, these differences may derive from differences in the amount or affinity of the cognate binding proteins for their target sites in the replicator. In a recent study an unexpectedly large number of transcription factor binding site regions were predicted to exist within the genome, with roughly 50% of these not associated with RNA coding genes (16). Taken together with the results presented here, these data lead us to speculate that transcription factor binding at genomic sites not associated with canonical promoter elements may influence replicator activity. Experiments are under way to determine the effects of other transcriptional activators and repressors on the c-*myc* replicator.

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