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A test of the model that RNA polymerase III transcription is regulated by selective induction of the 110 kDa subunit of TFIIIC

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ABSTRACT

TFIIIC is a RNA polymerase (pol) III-specific DNA-binding factor that is required for transcription of tRNA and 5S rRNA genes. Active human TFIIIC consists of five subunits. However, an inactive form has also been isolated that lacks one of the five subunits, called TFIIIC110. A model was proposed in which pol III transcription might be regulated by the specific induction of TFIIIC110, allowing formation of active TFIIIC from the inactive form. We have tested this model by transient transfection of HeLa and HEK293 cells with a vector expressing TFIIIC110. We have also made stably transfected HeLa cell lines that carry a doxycycline-inducible version of the cDNA for TFIIIC110. We show that the induced TFIIIC110 enters the nucleus, binds other TFIIIC subunits and is recruited to tRNA and 5S rRNA genes *in vivo*. However, little or no effect is seen on the expression of pol III transcripts. The data argue against the model that pol III transcription can be effectively modulated through the specific induction of TFIIIC110.

INTRODUCTION

TFIIIC is a large DNA-binding factor that directly recognizes the promoter sequences found within most pol III-transcribed genes, including tRNA and 7SL RNA genes and the adenoviral VA genes (1–3). It is also required for transcription of 5S rRNA genes, although in this case its recruitment is dependent on an additional polypeptide called TFIIIA (1–3). Indeed, it is probable that TFIIIC is used by all pol III templates in the yeast *Saccharomyces cerevisiae*, although it is not necessary for pol III transcription of mammalian U6 and 7SK RNA genes (1–3).

Human TFIIIC has been purified from HeLa cells as a complex of five subunits (4–6). These are commonly referred to as TFIIIC220, TFIIIC110, TFIIIC102, TFIIIC90 and TFIIIC63, according to their approximate molecular masses. An alternative nomenclature of TFIIICα, β, γ, δ and ε has also been used (7). The complex is stable, as the subunits remain associated following immunoprecipitation and washing in 1 M NaCl (8). Cloning of cDNAs for all five subunits has allowed confirmation that each is a bona fide component of TFIIIC (6,8–11). However, the complex has also been purified with the TFIIIC110 subunit missing (5). Furthermore, calculations based on metabolic labeling suggested that TFIIIC from cycling HeLa cells contains half as much TFIIIC110 as TFIIIC220 (7). TFIIIC complexes with or without the 110 kDa subunit were dubbed TFIIIC2a and TFIIIC2b, respectively, and shown to be resolved on non-denaturing gels or by gradient chromatography (5,9,12). Although the two forms produced identical footprints and had similar DNA-binding affinities, the complex without TFIIIC110 was unable to support transcription (5,12). These observations lead to a model in which TFIIIC activity could be regulated by reversible interaction of the TFIIIC110 subunit with the remainder of the complex (Figure 1). The interaction might be controlled by phosphorylation, as TFIIIC2b could be generated by phosphatase treatment of fractions containing active TFIIIC2a (12). The model was proposed to explain pol III transcriptional induction observed when HeLa cells are stimulated with serum or with adenoviral E1A (5,9,12).

The E1A oncoprotein of adenovirus can stimulate pol III transcription, probably through several complementary mechanisms (5,9,12–20). Hoeffler *et al*. (12) reported that the ratio of active TFIIIC2a to inactive TFIIIC2b, as measured by electrophoretic mobility shift assay (EMSA), is higher in HeLa cells infected with wild-type adenovirus than in cells infected with the E1A deletion mutant dl312. In support of this, western blotting revealed lower levels of TFIIIC110 following dl312 infection, compared with wild-type virus (9). EMSA analysis also suggested that the ratio of TFIIIC2a to TFIIIC2b is elevated in the E1A-transformed

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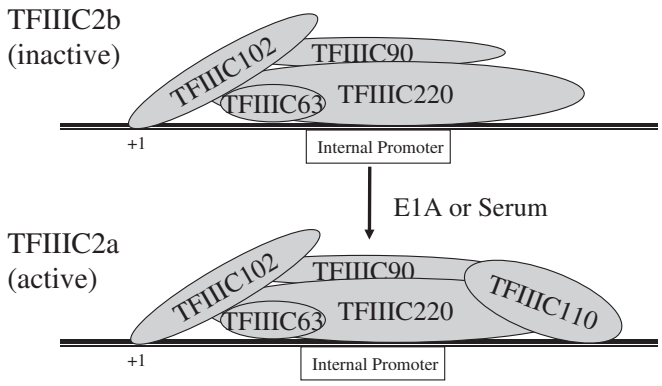

Figure 1. Model proposing that an inactive form of TFIIIC, called TFIIIC2b, is converted to an active form, called TFIIIC2a, by the selective induction of TFIIIC110 in response to serum or E1A. Both forms bind to promoter DNA, but only TFIIIC2a is competent to support transcription.

human embryonic kidney cell line 293 and in two SV40-transformed lines of murine fibroblasts (12,21). Subsequent studies found that all five subunits of TFIIIC are overexpressed in the SV40-transformed fibroblasts compared to untransformed parental cells, although the effect appears strongest for TFIIIC110 (22,23).

The rate of synthesis of pol III products is influenced strongly in mammalian cells by serum availability. Hoeffler *et al*. (12) reported that the relative proportion of TFIIIC2a to TFIIIC2b was reduced in HeLa cells grown in low serum, an effect which correlated with the reduced transcriptional activity. Consistent with this, western blotting revealed a decrease in expression of TFIIIC110 under low serum conditions, whereas TFIIIC220 levels were unchanged (9). A selective change in TFIIIC110 expression was therefore proposed as a mechanism allowing the pol III machinery to adapt to serum availability.

The model in Figure 1 was based on correlative data—relatively high ratios of TFIIIC2a to TFIIIC2b and of TFIIIC110 to TFIIIC220 seen in transcriptionally active extracts from cells grown in the presence of E1A or high serum. However, these observations did not distinguish between cause and effect. The data are compatible with the possibility that selective loss of TFIIIC110 might occur as a consequence of down-regulated transcription, rather than having a causative role. For example, surplus TFIIIC might be degraded when active transcription is not occurring and TFIIIC110 might simply be less stable than the other subunits and therefore removed first, allowing accumulation of TFIIIC2b. Therefore, we tested the model directly by determining whether a specific induction of TFIIIC110 is sufficient to stimulate pol III transcription.

MATERIALS AND METHODS

Cell culture

HeLa and HEK293 cells were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. HeLa TET-ON cells were cultured in 10% FBS (tetracycline free), 100 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml G418. Stably transfected HeLa TET-ON cells were also supplemented with 100 μg/ml hygromycin. Expression of HA-TFIIIC110 and HA-Brf1 was induced by the addition of 1 μg/ml doxycycline for 48 h.

HeLa cell proliferation assay

Cells were plated on to 75 cm^2 flasks at a density of 5×10^5 cells per plate, in media containing 0.5% or 10% FBS. Viable cells were counted every 24 h using trypan blue staining and a haemocytometer.

Transient and stable transfection

Transient transfection of HeLa and HEK293 cells were carried out using Lipofectamine. Stable transfection of HeLa TET-ON cells were also achieved using Lipofectamine. Stable transformants were selected using 200 μg/ml hygromycin.

Plasmids

pVA1 contains the adenovirus VA1 gene (24). pEGFP (Clontech) contains a cDNA encoding a derivative of green fluorescent protein expressed from a cytomegalovirus (CMV) promoter. pTRE2hygHA-Brf1 was produced by PCR amplification and sub-cloning into the pTRE2hyg vector (Clontech) the HA-Brf1 coding sequence from pcDNA3HA-Brf1 (25). Human TFIIIC110 was PCR amplified from the pRSET-TFIIIC110 vector (a kind gift from Robert Roeder) and subcloned into pcDNA3HA, generating pcDNA3HA-TFIIIC110. HA-TFIIIC110 was subsequently subcloned into pTRE2hyg, again by PCR, to generate pTRE2hygHA-TFIIIC110.

Western blotting and antibodies

Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and scraped into lysis buffer [20 mM HEPES (pH 7.8), 150 mM NaCl, 25% glycerol, 50 mM NaF, 0.2 mM EDTA, 0.5% Triton X-100, 0.5% NP-40, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethlysulfonyl fluoride (PMSF), 1 mM DTT, 0.5 μg/ml leupeptin, 1.0 μg/ml trypsin inhibitor, 0.5 μg/ml aprotinin and 40 μg/ml bestatin]. Lysates were incubated on ice for 10 min and passed through a 26-gauge needle three times, before centrifugation at 16 000 G for 10 min at 4°C. The supernatant was collected for immunoblot analysis, which was performed as described previously (26).

Antibodies were F-7 against HA, SI-1 against TFIIB, C11 against actin and R-124 against cyclin D1 from Santa Cruz Biotechnologies. The Rb antibody G3-245 was from BD Pharmingen. Peptide antisera 128 against Brf, Ab4 against TFIIIC220, MTBP-6 against TBP and 1898 against TFIIIC90 have been described previously (7,23,25,27,28). Antibody 3208 against TFIIIC110 was raised by immunizing rabbits with synthetic peptide GEAGPVGNMTVVDSP (human TFIIIC110 residues 12–26) coupled to keyhole limpet haemocyanin.

Immunoprecipitation

Whole cell extracts (150 μg), prepared as described previously (26), were incubated in an orbital shaker for 3 h at 4°C with 30 μl of protein G beads carrying equivalent

amounts of pre-bound IgG. Samples were pelleted and the beads washed five times with PBS. The bound material was analysed by western blotting.

Primer extension

Total RNA was extracted using TRI reagent, according to manufacturers' instructions. RNA was then analysed by primer extension using labelled primers specific for VA1 5′-CACGCGGGCGGTAACCGCATG-3′ and green fluorescent protein (GFP) 5′-CGTCGCCGTCCAGCTCGACCAG-3′. Primer extension assays were performed as described previously (20).

RT–PCR

RT–PCR analysis of ARPP P0 mRNA, 5S rRNA and tRNA transcripts were performed as described previously (29,30).

Northern blotting

Northern blotting was carried out as described previously (28). The ARPP P0 probe was a 1 kb EcoRI–HindIII fragment from the mouse cDNA (31). The tRNA probe was a 240 bp EcoRI–HindIII fragment from pLeu (32).

Immunofluorescence microscopy

Cells on coverslips were fixed in 4% para-formaldehyde in PBS for 10 min, followed by permeabilization in 0.1% Triton X-100 in PBS for 4 min. Cells were washed, blocked in 0.1% goat serum and 0.2% fish skin gelatin in PBS, before incubation with primary antibody (HA from Santa Cruz) at 1:100 or 1:500 dilution. Cells were again washed and blocked followed by incubation with secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, from Molecular Probes) at 1:400 dilution. Cells were washed with PBS and mounted on to glass slides and viewed using a Zeiss Axiovert fluorescence microscope.

Chromatin immunoprecipitation (ChIP)

ChIP assays were carried out as described (33). The antibodies used were MTBP-6 against TBP (27), 4286 against TFIIIC110 (25), F-7 against HA and SI-1 against TFIIB (both from Santa Cruz Biotechnologies). Primers and cycling parameters were as described previously (29,33). Serial dilutions of chromatin were used to confirm PCRs were within a linear range.

RESULTS

Transient transfection of a TFIIIC110 expression vector has minimal effect on pol III transcription in HeLa and HEK293 cells

A mammalian expression vector was constructed that allows overexpression of an mRNA encoding HA-tagged TFIIIC110. This construct was transiently transfected into HEK293 cells along with the adenoviral VA1 gene, the pol III-transcribed gene used in the original studies by Hoeffler, Kovelman and Roeder (5,12). A vector encoding GFP transcribed from the CMV promoter was cotransfected as a control for transfection efficiency. Western blotting for the HA tag confirmed that the exogenous TFIIIC110 protein

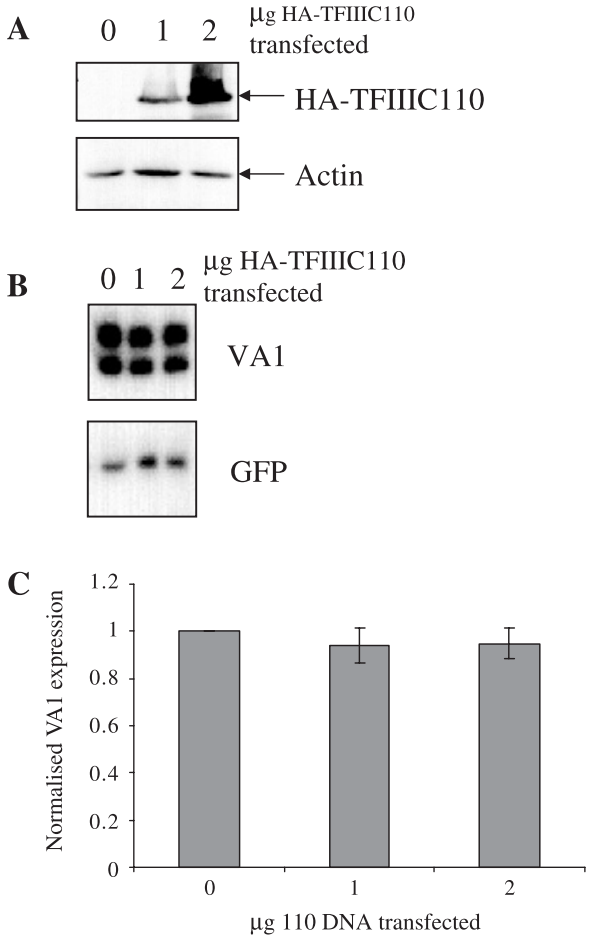

Figure 2. Transient transfection of a HA-TFIIIC110 expression vector has little or no effect on VA1 transcript levels in HEK293 cells. (**A**) Western blot of HEK293 cells transfected with pcDNA3HA-TFIIIC110 and/or pcDNA3-HA to a total concentration of 2 μg. Upper panel shows a blot for HA-TFIIIC110, lower panel shows a blot for actin. (**B**) Primer extension analysis of VA1 and GFP RNA in HEK293 cells transfected with 0.25 μg of each of the VA1 and GFP plasmids and with pcDNA3HA-TFIIIC110 and/or pcDNA3HA to a total concentration of 2 μg. (**C**) Quantitation of the data from (B) and three identical experiments.

was expressed in the transfected cells (Figure 2A). However, little or no effect was seen in multiple experiments on the level of VA1 transcript, as assayed by primer extension (Figure 2B and C).

The lack of response in HEK293 cells might be explained by the fact that this line expresses E1A, which was reported to elevate TFIIIC110 expression; the ratio of this polypeptide with respect to other TFIIIC subunits may already be high in these cells, such that TFIIIC2a predominates (12). Therefore, we repeated the experiment in HeLa cells, which were reported to have a significant fraction of TFIIIC2b, which lacks the TFIIIC110 subunit. In contrast to HEK293 cells, a modest, dose-dependent induction of VA1 RNA was observed when the HA-TFIIIC110 expression vector was transfected into HeLa cells (Figure 3A and B). After normalization to the cotransfected GFP internal control, the average response was ~1.5-fold (Figure 3C).

RT–PCR analysis was used to determine if levels of the endogenous pol III transcripts are influenced by transient

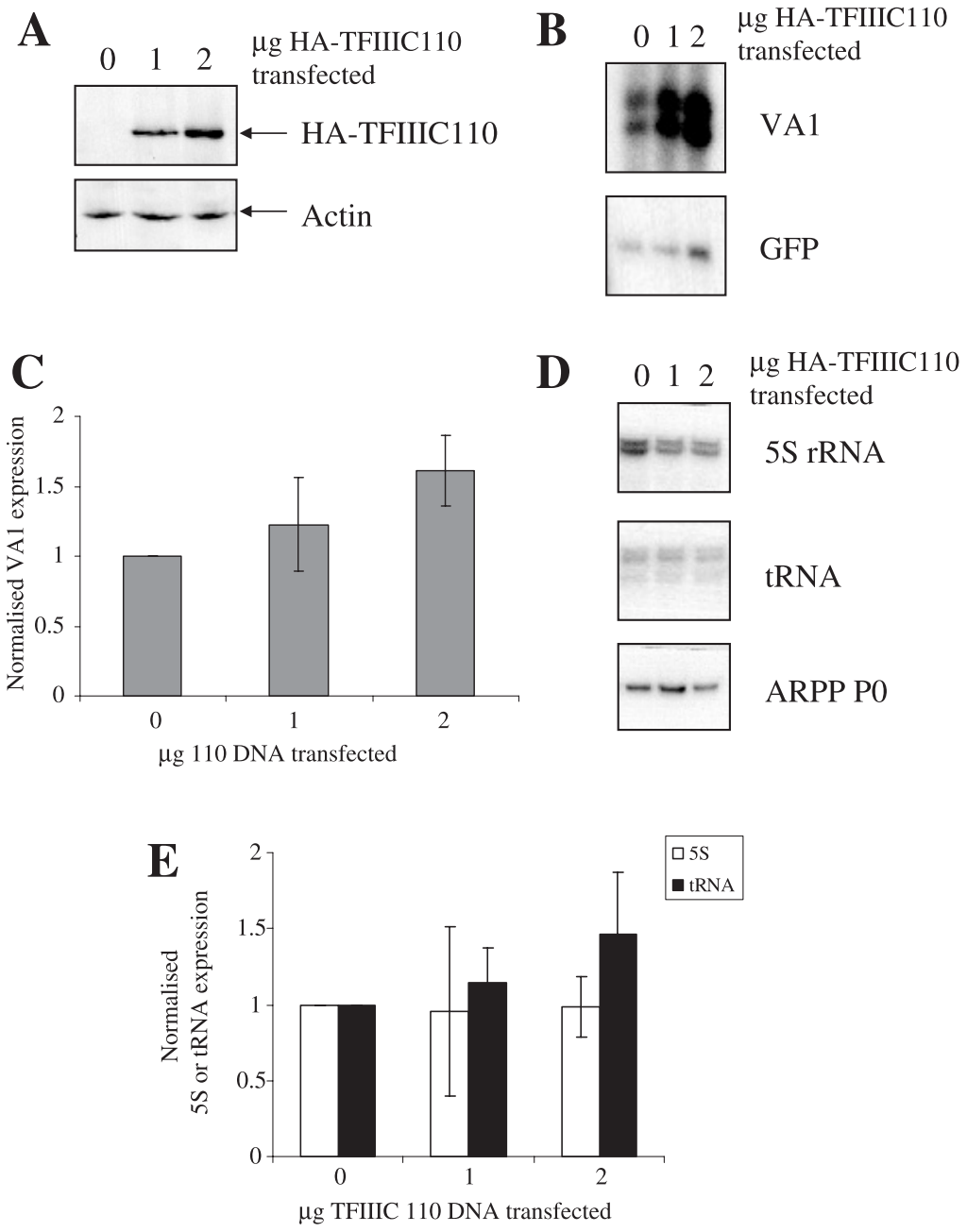

Figure 3. Transient transfection of a HA-TFIIIC110 expression vector has only a minor effect on VA1 reporter or endogenous pol III transcript expression in HeLa cells. (**A**) Western blots of HeLa cells transfected with pcDNA3HA-TFIIIC110 and/or pcDNA3HA to a total concentration of 2 µg. Upper panel shows a blot for HA-TFIIIC110, lower panel shows a blot for actin. (**B**) Primer extension analysis of VA1 and GFP RNA in HeLa cells transfected with 0.25 µg of each of the VA1 and GFP plasmids and with pcDNA3HA-TFIIIC110 and/or pcDNA3HA to a total concentration of 2 µg. Note that lane 3 is somewhat overloaded. (**C**) Quantitation of the data from Figure 3B and three additional experiments. (**D**) RT–PCR analysis of 5S rRNA, tRNA and ARPP P0 mRNA, as indicated, in HeLa cells transfected with pcDNA3HA-TFIIIC110 and/or pcDNA3HA to a total concentration of 2 µg. (**E**) Quantitation of the data from (D) and three additional experiments.

transfection of the HA-TFIIIC110 vector. We examined expression of 5S rRNA and tRNA genes, both of which require TFIIIC for their transcription (1–3). No reproducible induction was observed for 5S rRNA, whereas tRNA showed a slight response of ~1.5-fold (Figure 3D and E). Although, this assay measures the steady-state level of 5S rRNA, our tRNA primers are specific for short-lived primary transcripts, which are considered to reflect the rate of ongoing transcription (29). This approach therefore provided limited support for the model that class III gene expression can be stimulated by a specific increase in the ratio of TFIIIC110 to other TFIIIC subunits.

Stable induction of TFIIIC110 in HeLa cells has little effect on expression of tRNA and 5S rRNA

The experiments in Figure 3D and E measured levels of endogenous transcripts in the total cell population. However,

immunofluorescence analysis of GFP expression from the cotransfected control vector indicated that many of the HeLa cells remain untransfected under the conditions of our assays (data not shown). Since the presence of untransfected cells may partially mask a response, we produced HeLa cell clones that were stably transfected in the presence of a selectable marker, to ensure that the entire population receives the expression vector. The TFIIIC110 cDNA was subcloned into pTRE2hyg, which carries a doxycycline-responsive promoter. This was then used to produce multiple clones in which HA-tagged TFIIIC110 can be induced specifically by addition of doxycycline to the culture medium. Individual clones varied considerably in the levels of HA-TFIIIC110 that were produced (Figure 4A). We also observed clonal variation in the expression of tRNA and 5S rRNA (Figure 4B). However, RT–PCR analysis revealed no consistent change in the expression of pol III transcripts following induction of HA-TFIIIC110 (Figure 4B and C).

The model in Figure 1 predicts that pol III transcription might be more sensitive to TFIIIC110 induction under low serum conditions. Therefore, we cultured HeLa cells in 0.5% serum, as in the previous work (9,12). This treatment was sufficient to cause a marked reduction in cell proliferation (Figure 5A). Trypan blue staining did not reveal any substantial apoptosis (data not shown). As expected (32,34,35), tRNA expression was markedly diminished in low serum (Figure 5B). This was accompanied by a slight decrease in expression of endogenous TFIIIC110, as revealed by western blotting (Figure 5C). HA-TFIIIC110 remained inducible under these conditions (Figure 5D), but we again found that its induction has little or no effect on tRNA or 5S rRNA levels (Figure 5E and F).

Control experiments were conducted to verify that the exogenous HA-TFIIIC110 is incorporated into TFIIIC complexes. Sequence analysis using the PSORT II programme (http://psort.ims.u-tokyo.ac.jp) revealed a strong nuclear localization signal (NLS) in TFIIIC220, but not in the other TFIIIC subunits. Unincorporated HA-TFIIIC110 may therefore be retained in the cytoplasm. Indeed, cytoplasmic fluorescence was detected with antibody against the HA tag following addition of doxycycline to the stable transfectants (Figure 6A). However, an intense immunofluorescence signal was also observed from the nuclei. This is consistent with the possibility that although some of the induced protein may remain unincorporated and hence in the cytoplasm, a significant proportion associates with TFIIIC220 to form a complex that enters the nucleus. Complex assembly is supported by the finding that HA-TFIIIC110 can be coimmunoprecipitated from doxycycline-treated cells using antiserum against TFIIIC220 (Figure 7A). Other TFIIIC subunits also associate with the HA-TFIIIC110, as shown by the fact that anti-HA antibody coimmunoprecipitates TFIIIC90 following doxycycline treatment (Figure 7B). This interaction is specific, since TFIIIC90 is not detected after control immunoprecipitations using antibodies against RB or cyclin D1. In addition, HA-TFIIIC110 is detected by ChIP at chromosomal tRNA and 5S rRNA genes, but not at the gene encoding TFIIIC220, which we used as a pol II-transcribed negative control (Figure 7C). In contrast, the pol II-specific transcription factor TFIIB is detected at the TFIIIC220 gene, but not at tRNA or 5S rRNA genes, as expected. These data confirm

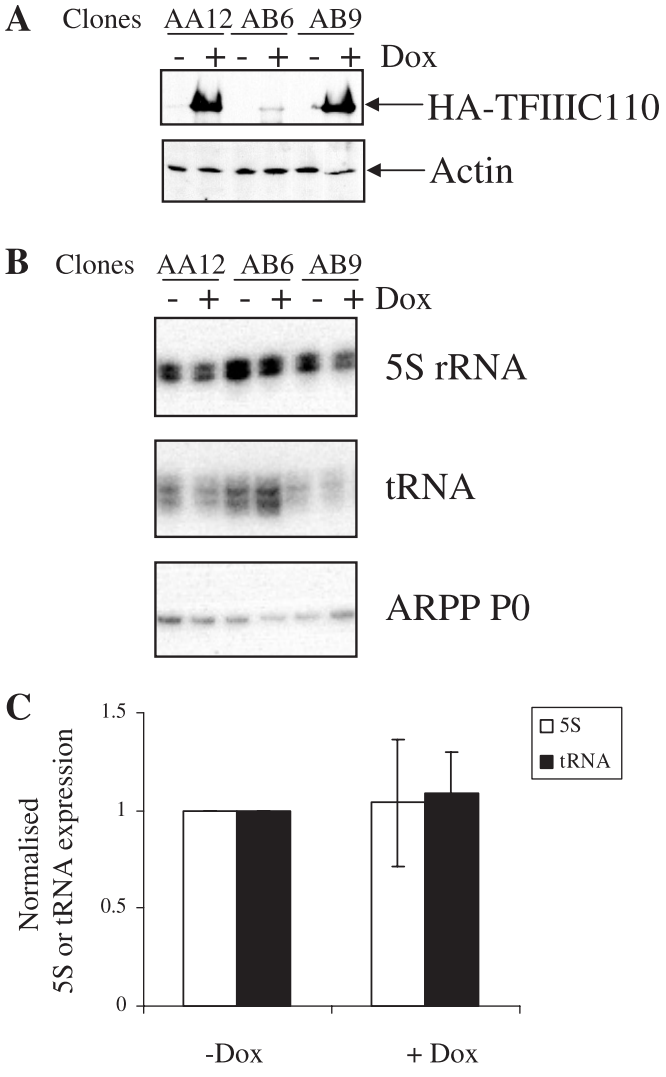

Figure 4. Stable expression of HA-TFIIIC110 has little or no effect on endogenous pol III transcript levels in actively proliferating HeLa cells. (**A**) Western blot of HeLa cell clones stably transfected with pTRE2hygHA-TFIIIC110 and cultured in 10% serum in the presence or absence of doxycycline for 48 h. Upper panel shows a blot for HA-TFIIIC110, lower panel shows a blot for actin. (**B**) RT–PCR analysis of 5S rRNA, tRNA and ARPP P0 mRNA, as indicated, in HeLa cells stably transfected with pTRE2hygHA-TFIIIC110 and grown in 10% serum in the presence or absence of doxycycline for 48 h. (**C**) Quantitation of the data from three independent HeLa cell clones stably transfected with pTRE2hygHA-TFIIIC110 and grown in 10% serum in the presence or absence of doxycycline.

that the doxycycline-induced exogenous HA-TFIIIC110 is incorporated into TFIIIC complexes that are recruited to the appropriate target genes *in vivo*. However, ChIP with an antibody that recognizes total TFIIIC110 (i.e. both endogenous and HA-tagged forms), shows no increase in gene occupancy in response to doxycycline (Figure 7C, lanes 3 and 4). The data suggest that although stable transfection with an inducible construct is able to raise the total level of TFIIIC110 in HeLa cells, it has little impact on the amount of this subunit that is assembled onto promoters. This is consistent with the lack of a significant transcriptional response.

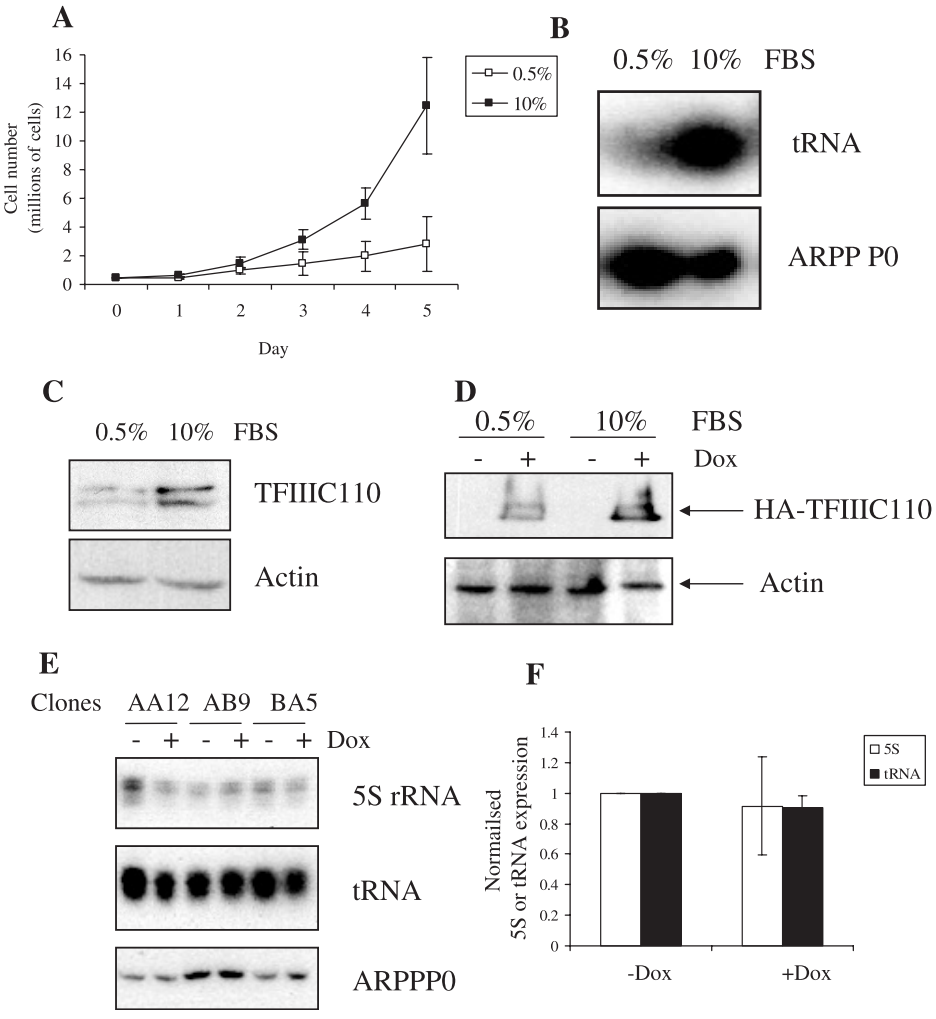

Figure 5. Stable expression of HA-TFIIIC110 has little or no effect on endogenous pol III transcript levels in HeLa cells cultured in low serum. (**A**) Proliferation curve of HeLa cells cultured for 7 days in media containing 0.5 or 10% serum. (**B**) Northern blot analysis of total RNA (10 μg) extracted from HeLa cells cultured for 4 days in media containing 0.5 or 10% serum. The upper panel shows the blot probed with a $tRNA^{Leu}$ gene and the lower panel shows the same blot, which has been stripped and re-probed for ARPP P0 mRNA. (**C**) Western blot of endogenous TFIIIC110 and actin in HeLa cells cultured for 4 days in media containing 0.5 or 10% serum. (**D**) Western blot of HeLa cells stably transfected with pTRE2hygHA-TFIIIC110 and cultured in 0.5 or 10% serum in the presence or absence of doxycycline for 48 h. Upper panel shows a blot for HA-TFIIIC110, lower panel shows a blot for actin. (**E**) RT–PCR analysis of 5S rRNA, tRNA and ARPP P0 mRNA, as indicated, in HeLa cells stably transfected with pTRE2hygHA-TFIIIC110 and cultured in 0.5% serum in the presence or absence of doxycycline for 48 h. (**F**) Quantitation of the data from three independent HeLa cell clones stably transfected with pTRE2hygHA-TFIIIC110 and cultured in 0.5% serum in the presence or absence of doxycycline.

Stable induction of Brf1 in HeLa cells can stimulate expression of tRNA and 5S rRNA

The data above show that specific induction of TFIIIC110 in HeLa cells has minimal effect on the expression of endogenous tRNA and 5S rRNA, even though the induced protein interacts with other TFIIIC subunits and is recruited to the appropriate template genes. Since this can be viewed as a negative result, it was necessary to confirm that the system we have used is amenable to transcriptional induction if a limiting factor is employed. We chose to try the Brf1 subunit of TFIIIB, as this has been shown to stimulate tRNA and 5S rRNA gene transcription when titrated into HeLa cell extracts. Therefore, we prepared stably transfected HeLa cell clones that carry doxycycline-inducible HA-tagged Brf1, using exactly the same approach as described above for HA-TFIIIC110. Blotting for the HA tag confirmed that HA-Brf1 can be induced in these cells by the addition of doxycycline (Figure 8A). A high proportion of this protein is localized to the nucleus (Figure 6B). Furthermore, RT–PCR analysis showed that tRNA and 5S rRNA levels are increased significantly in response to induction of HA-Brf1 (Figure 8B and C). This confirms that the doxycycline-regulated system used in these experiments can be exploited to activate pol III transcription. Therefore, we infer that a specific increase in the level of TFIIIC110 may not be an effective mechanism for pol III regulation in HeLa cells, as postulated.

DISCUSSION

In these experiments we have tested directly the model that TFIIIC110 is ‘a central controlling subunit’ for pol III

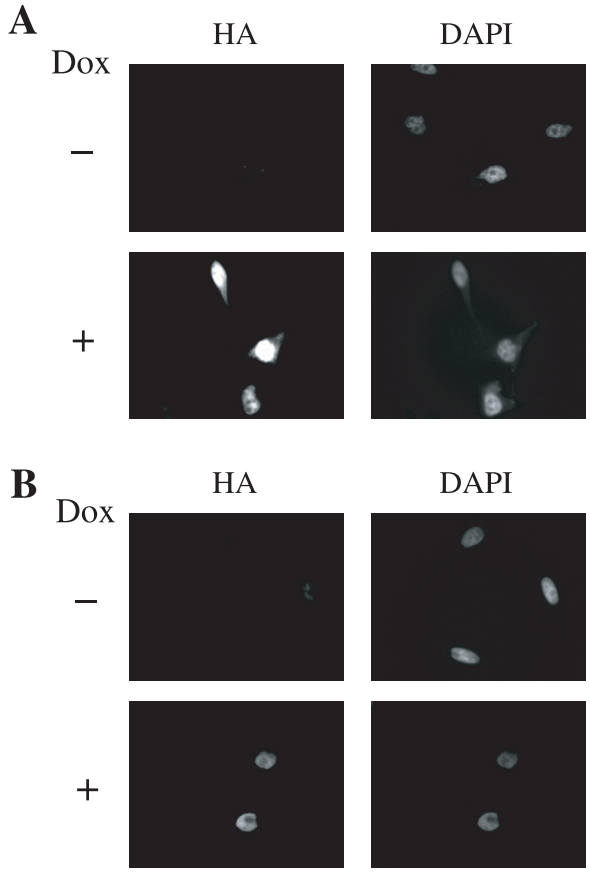

Figure 6. Immunofluorescence analysis of the subcellular distribution of HA-TFIIIC110 and HA-Brf1. Left panels show the localization of HA antigen in cells stably transfected with pTRE2hygHA-TFIIIC110 (**A**) or pTRE2hygHA-Brf1 (**B**) and cultured without doxycycline (upper panels) or induced with doxycycline for 48 h (lower panels). Right panels show DAPI staining of the same cells.

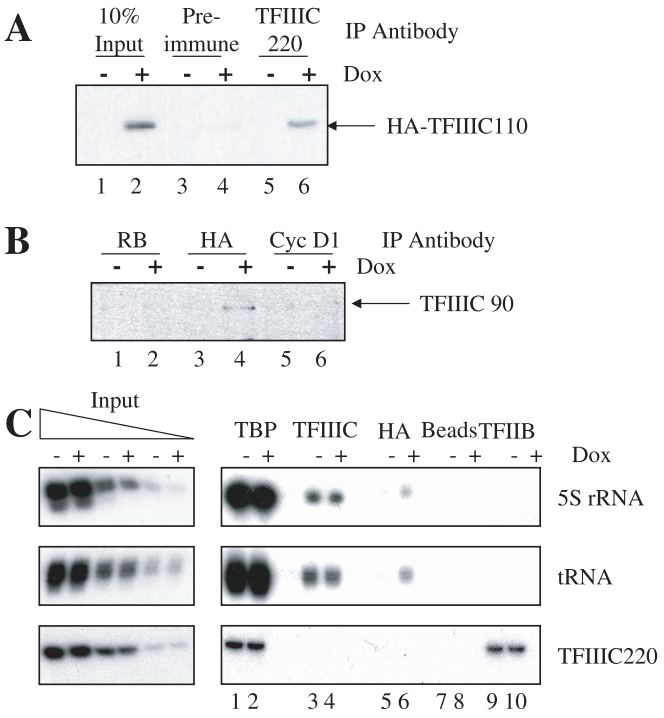

Figure 7. HA-TFIIIC110 associates with other TFIIIC subunits and pol III-transcribed genes. (**A**) Western blot using anti-HA antibody of material immunoprecipitated using preimmune serum (lanes 3 and 4) or antiserum against TFIIIC220 (lanes 5 and 6), as well as 10% inputs (lanes 1 and 2), from pTRE2hygHA-TFIIIC110 stably transfected HeLa cells cultured in the absence (odd-numbered lanes) or presence (even-numbered lanes) of doxycycline. (**B**) Proteins immunoprecipitated using monoclonal antibodies against RB (lanes 1 and 2), HA (lanes 3 and 4) and cyclin D1 (lanes 5 and 6) from pTRE2hygHA-TFIIIC110 stably transfected HeLa cells cultured in the absence (odd-numbered lanes) or presence (even-numbered lanes) of doxycycline were western blotted with antiserum against TFIIIC90. (**C**) ChIP assay using pTRE2hygHA-TFIIIC110 stably transfected cells cultured in the absence or presence of doxycycline, as indicated. Cross-linked chromatin was immunoprecipitated using antibodies against TBP, TFIIIC110, HA or TFIIB, or was mock-immunoprecipitated with beads unattached to antibody, as indicated. Immunoprecipitated material was PCR amplified using primers to the genes encoding 5S rRNA, $tRNA^{Leu}$ and TFIIIC220, as indicated. Input panels show product intensities obtained using 10, 2 and 0.4% of input.

transcription of TFIIIC-dependent promoters, as suggested previously (9). Our data do not support this idea. A weak induction by TFIIIC110 was observed for the VA1 and tRNA genes in transiently transfected cells, but this was only around 1.5-fold. Furthermore, this small effect was not reproducible in stably transfected cells. 5S rRNA gene expression did not respond to TFIIIC110 induction under either circumstance. In contrast, induction of Brf1 produces a strong increase in tRNA and 5S rRNA expression under the same conditions. We have confirmed that the induced TFIIIC110 can interact with other TFIIIC subunits, enter the nucleus and be recruited specifically to target genes. However, this has little effect on the overall amounts of TFIIIC110 being recruited to chromosomal genes. The data suggest that this subunit is not limiting in the contexts we have investigated.

It was reported that several unidentified polypeptides co-purified with inactive TFIIIC2b and were not associated with active TFIIIC2a (5,9). In particular, a polypeptide of 77 kDa co-fractionated consistently with TFIIIC2b, although it was never detected by co-immunoprecipitation (9). As it was unclear whether this was a contaminant or had functional significance, the model of TFIIIC regulation by differential interaction with TFIIIC110 included the possibility that TFIIIC2b might contain an additional 77 kDa subunit as well as lacking TFIIIC110 (9). As far as we are aware, the identity of this putative subunit has not been reported. Clearly, the possibility of its involvement has not been addressed by our study.

Experiments carried out by Shen *et al*. lead them to question the existence of TFIIIC2b (7). These authors raised an antiserum against TFIIIC110 that, when used in an EMSA, was able to block all the DNA-binding activity of TFIIIC in a HeLa extract (7). This finding clearly conflicts with the report that a significant fraction of HeLa cell TFIIIC exists in a form (i.e. TFIIIC2b) that lacks TFIIIC110 but is undiminished in its DNA-binding capacity (5,12). We have been unable to address this issue because our antisera against TFIIIC110 are unable to block or supershift in an EMSA. Our study has specifically examined the hypothesis that selective induction of TFIIIC110 is a significant regulatory mechanism *in vivo*.

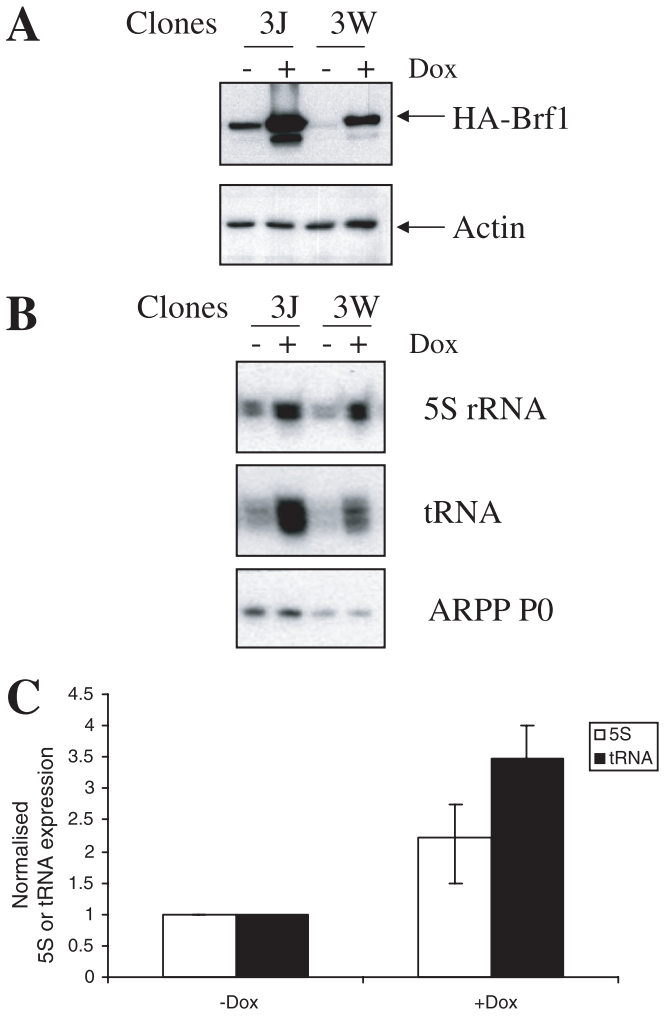

Figure 8. Induction of HA-Brf1 can stimulate expression of tRNA and 5S rRNA genes in stably transfected HeLa cells. (**A**) Western blot of HeLa cell clones stably transfected with pTRE2hygHA-Brf1 and grown in the presence or absence of doxycycline for 48 h. The upper panel shows a blot for HA and the lower panel shows a blot for actin. (**B**) RT–PCR analysis of 5S rRNA, tRNA and ARPP P0 mRNA, as indicated, in HeLa cells stably transfected with pTRE2hygHA-Brf1 and grown in 10% serum in the presence or absence of doxycycline for 48 h. (**C**) Quantitation of the data shown in (B).

Another unresolved question with regard to mammalian TFIIIC concerns the identity of TFIIIC1, a TFIIIC-associated fraction that was found to be required for transcription of all types of pol III template using partially-purified reconstituted systems (36–41). Although candidate components of TFIIIC1 have been suggested (9,41), the functional significance of these polypeptides has not been demonstrated, as far as we are aware. Indeed, efficient pol III transcription of a U6 gene has been reconstituted more recently using a combination of recombinant and highly purified proteins without any TFIIIC1 fraction (42). It was suggested that TFIIIC1 might in fact correspond to Bdp1, an essential subunit of TFIIIB that dissociates readily during purification (42). This possibility is supported by the finding that Bdp1 co-fractionates with TFIIIC1 activity and can substitute for TFIIIC1 fractions in a reconstituted transcription assay (43).

The model of TFIIIC110 induction was proposed as a mechanism to explain the activation of pol III transcription by E1A and by serum (5,9,12). We have not examined whether E1A can selectively stimulate TFIIIC110 expression, but our data suggest that this would have little impact on transcriptional output, at least in HeLa cells. Other documented mechanisms may instead account for pol III activation under these circumstances. Yoshinaga *et al.* (16) demonstrated that overall levels of TFIIIC had increased 4- to 8-fold 30 h after adenovirus infection of HeLa cells. In addition, E1A can overcome RB-mediated repression of pol III transcription, both *in vitro* and *in vivo* (20). Serum stimulation leads to a phosphorylation-mediated inactivation of RB and so can also derepress the pol III machinery (44). Furthermore, pol III transcription is directly activated by c-Myc and Erk, both of which are serum-inducible (33,45). Combinations of these mechanisms may be sufficient to explain pol III responsiveness to E1A and to serum.

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