TRRAP and GCN5 are used by c-Myc to activate RNA polymerase III transcription


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Activation of RNA polymerase (pol) II transcription by c-Myc generally involves recruitment of histone acetyltransferases and acetylation of histones H3 and H4. Here, we describe the mechanism used by c-Myc to activate pol III transcription of tRNA and 5S rRNA genes. Within 2 h of its induction, c-Myc appears at these genes along with the histone acetyltransferase GCN5 and the cofactor TRRAP. At the same time, occupancy of the pol III-specific factor TFIIIB increases and histone H3 becomes hyperacetylated, but increased histone H4 acetylation is not detected at these genes. The rapid acetylation of histone H3 and promoter assembly of TFIIIB, c-Myc, GCN5, and TRRAP are followed by recruitment of pol III and transcriptional induction. The selective acetylation of histone H3 distinguishes pol III activation by c-Myc from mechanisms observed in other systems.

acetyltransferase | chromatin | histone | TFIIIB | TFIIIC

The protooncogene product c-Myc is a potent inducer of cell growth and proliferation (1, 2), which it achieves through complex transcriptional programs involving large numbers of target genes (1, 2). Many of these targets encode components of the ribosome (3). Thus, c-Myc induces transcription by RNA polymerase (pol) II of many ribosomal protein genes and can also stimulate the synthesis of tRNA and rRNA, by directly activating transcription by pols I and III (3, 4). This striking ability to activate transcription by three different pols allows c-Myc to induce multiple components of the ribosome coordinately and thereby stimulate a cell’s capacity for protein synthesis and hence growth.

The discovery that pol III-transcribed genes are targets for c-Myc was unexpected because their promoters do not usually contain good matches to the E-box DNA sequence that is characteristically used by c-Myc to activate transcription by pols I and II. Nevertheless, a hydroxymethylxen (OHT)-inducible Myc-estrogen receptor (MycER) fusion will specifically induce pol III-transcribed genes in human diploid fibroblasts and transgenic mice (4, 5). The rapidity of this response suggests direct regulation, which is supported by experiments in which α-amanitin was used to exclude pol II-mediated effects (4). Direct action was confirmed by ChIP, which showed that endogenous c-Myc associates with tRNA and 5S rRNA genes in vivo (4, 6). The pol III-specific transcription factor TFIIIB was found to bind in vitro to the c-Myc transactivation domain, while communoprecipitation and cofractionation experiments showed that endogenous c-Myc associates stably with endogenous TFIIIB (4). Furthermore, scanning ChIP assays suggest that c-Myc colocalizes with TFIIIB in the vicinity of the transcription start site [supporting information (SI) Fig. 6]. These observations support the hypothesis that it is TFIIIB, rather than a DNA sequence, which attracts c-Myc to pol III templates.

How pol III transcription is induced remains to be determined. Most recent work on the mechanisms of activation by c-Myc has focused on its ability to influence chromatin structure (7–9). The transactivation domain of c-Myc binds to the cofactor TRRAP (10), a 400-kDa member of the ATM family that forms complexes with the histone acetyltransferases (HATs) GCN5, p300/CBP-associated factor (PCAF), and TIP60 (11, 12). These interactions allow c-Myc to recruit HAT activity to the vicinity of E-box sites, leading to acetylation of histones H3 and H4 (13–16). Binding has also been observed between c-Myc and the INI1/hSNF5 subunit of the SWI/SNF chromatin remodeling complex and the ATPase/helicases TIP48 and TIP49 (17, 18). Indeed, c-Myc can influence nuclear condensation and histone modification across substantial domains (19, 20). Such effects on chromatin organization are likely to impact strongly on the expression of many genes.

The ability to induce chromatin structures that are conducive to transcription is considered a key feature of gene activation by c-Myc. However, it may not be universal, and other mechanisms have also been described. For example, activation of the cad promoter by c-Myc does not require recruitment of TRRAP and associated HATs and involves little change in histone acetylation (21, 22). In this case, c-Myc recruits the kinase P-TEFb, which may phosphorylate preassembled pol II and thereby stimulate promoter clearance and transcript elongation (22–24). A notable feature of this mechanism is that polymerase occupancy is already high at the uninduced cad promoter and does not increase further in response to c-Myc (22, 24). Activation of each of five other c-Myc targets examined also involved no apparent increase in the occupancy of pol II (24). In contrast, loading of P-TEFb, TFIIH, and Mediator increased at these promoters in response to c-Myc (24). Several earlier studies have shown association of c-Myc with TATA box-binding protein (TBP) through direct binding (25–28), providing another plausible mechanism for c-Myc to regulate gene expression. Indeed, substantial TBP recruitment by c-Myc is observed at pol I-transcribed rRNA genes (29). However, activation of the cyclin D2 promoter by c-Myc was not accompanied by any increase in TBP occupancy (24).

Here, we have characterized the molecular basis of pol III transcriptional activation by c-Myc. In some respects it resembles what has been previously described for pol II induction. For example, c-Myc recruits the cofactors TRRAP and GCN5 to tRNA and 5S rRNA genes and stimulates acetylation of histone H3 at these loci. In contrast, TIP60 is not detected and there is no evidence of increased histone H4 acetylation. Gene occu-
pency by TFIIB increases rapidly in response to c-Myc and is followed by recruitment of pol III.

Results

**TRRAP Associates with TFIIB and Activates Pol III Transcription.** To stimulate pol III transcription, c-Myc requires both its N-terminal transactivation domain (4) and its C-terminal dimerization and DNA-binding domain (SI Fig. 7). The pol III response is abolished by deletion of residues 106–143 from within the transactivation domain (4). This region includes the conserved MBII motif (residues 129–143), which is essential for cell transformation by c-Myc (30). Because MBII is the binding site for the transactivation/transformation domain-associated protein TRRAP (10), we used ChIP to test whether this cofactor is present at pol III-transcribed genes in vivo. Indeed, endogenous TRRAP was clearly detected at chromosomal tRNA and 5S rDNA genes, as was the TFIIB subunit Brf1, which was used as a positive control (Fig. 1A, B). In contrast, little or no binding was detected for the negative control TFIIB, which is pol II-specific. Furthermore, none of these proteins were found within the coding region of the gene encoding acidic ribosomal phosphoprotein (ARPP) P0. We conclude that TRRAP associates specifically with tRNA and 5S rDNA genes in vivo.

To test whether TRRAP influences pol III transcription, RNAi was used to reduce its expression. Relative to mock-transfected controls, levels of TRRAP mRNA and protein were specifically decreased when HeLa cells were transfected with siRNA against the message encoding TRRAP (Fig. 1B and C). In contrast, the TRRAP siRNA did not diminish expression of Oct1, actin proteins, or ARPP P0 mRNA. To evaluate the effect of TRRAP knockdown on pol III activity, we carried out RT-PCR using primers that recognize intron-containing pre-tRNA primary transcripts; as these products are processed very rapidly, their levels reflect the rate of transcription (31). Depletion of TRRAP was found to decrease expression of pre-tRNA (Fig. 1C), suggesting that pol III transcription is stimulated by TRRAP. No change in pre-tRNA level was seen in response to a control siRNA that efficiently depleted Oct-1. The response to TRRAP depletion is unlikely to be caused by fortuitous cross-reaction by the siRNA with an alternative target, as the same result was obtained with a vector encoding a specific short hairpin RNA against a different part of the TRRAP sequence (SI Fig. 8). We conclude that TRRAP has a positive effect on expression of pol III products.

**Recruitment of TRRAP to Pol III-Transcribed Genes Is Promoted by c-Myc.** ChIP assays were used to compare tRNA gene occupancy by TRRAP in wild type and c-Myc null fibroblasts. Expression of pre-tRNA is markedly diminished in the c-Myc<sup>−/−</sup> cells, when compared with the wild type (SI Fig. 9A). Consistent with this finding, pol III occupancy of the corresponding genes is lower in the knockouts, as assessed with antibody against the pol III subunit RPC155 (Fig. 2A). TRRAP occupancy is also decreased in the knockouts. This difference cannot be attributed to differences in expression, as levels of TRRAP and RPC155 are comparable in the two cell types (SI Fig. 9B). Instead, it appears that TRRAP recruitment is compromised in the c-Myc knockout cells.

Fibroblasts expressing an inducible MycER fusion construct were used to test whether c-Myc can recruit TRRAP to pol III templates. These cells were first starved of serum to deplete endogenous c-Myc. OHT triggers rapid accumulation within nuclei of the MycER fusion protein, as shown by Western blotting of nuclear extracts (SI Fig. 9C). No change was seen in the levels of representative subunits of TFIIB, TFIIC, and pol III. ChIP analysis shows that within 2 h of OHT addition MycER occupancy increases at tRNA and 5S rDNA genes (Fig. 2B and SI Fig. 9D). Similarly rapid recruitment is seen at the cyclin D2 promoter, a paradigm c-Myc target that contains an E-box DNA sequence. Nevertheless, binding is specific, as it is not detected
at the ARPP P0 gene. The data are clearly consistent with the pol III-transcribed genes serving as direct targets for c-Myc. Furthermore, TRRAP occupancy of these genes also increases within 2 h, in parallel with that of the MycER protein (Fig. 2C and SI Fig. 9E). Neither c-Myc nor TRRAP were recruited to 5S rRNA genes after OHT treatment of fibroblasts carrying empty vector instead of the MycER expression construct (data not shown). We conclude that c-Myc facilitates recruitment of TRRAP to pol III templates.

**GCN5 Activates Pol III-Transcribed Genes and Is Recruited in Response to c-Myc.** TRRAP has been shown to mediate recruitment by GCN5, TIP60, and OCT-1 (12). However, we found no binding to tRNA or 5S genes (data not shown). It therefore appears that only a subset of TRRAP-containing complexes is recruited to these pol III templates, particularly due to the stimulatory influence of GCN5 on pol III transcription (11). We therefore carried out ChIP assays to examine acetylation of histones at these loci (Fig. 4B and SI Fig. 11). As positive controls, we used the cyclin D2 promoter, where activation by c-Myc is known to involve histone acetylation (13). This site became enriched for acetylated histones H3 and H4 within 2 h of OHT addition to MycER-transduced fibroblasts. The response was specific, as it was not observed at the p21 gene promoter, which is not activated by c-Myc. As with the cyclin D2 promoter, acetylation of histone H3 was increased markedly at tRNA and 5S rRNA genes 2 h after MycER induction. In contrast, no increase in acetylated histone H4 was observed at these genes, either 2 or 4 h after induction. This finding is consistent with the failure of c-Myc to recruit TIP60 to these loci (Fig. 3D), as TIP60 is thought to be primarily responsible for H4 acetylation in response to c-Myc. Histone acetylation was not affected by OHT treatment of empty vector control cells (data not shown).

**Induction of c-Myc Triggers Selective Acetylation of Histone H3 at Pol III-Transcribed Genes.** The above results indicate that c-Myc recruits GCN5 to tRNA genes, we tested whether HAT activity contributes to pol III transcriptional activation. H3-CoA-20-Tat is a cell-permeable peptide CoA conjugate that specifically inhibits PCAF and its close relative GCN5 (34). This treatment was found to compromise tRNA gene induction when added to MycER fibroblasts before OHT addition (Fig. 4D). No such effect was observed when cells were treated in parallel with a related, but inactive, peptide control. These data suggest that the stimulatory influence of GCN5 on pol III transcription involves its HAT activity.

Because the above results indicate that c-Myc recruits GCN5 to tRNA genes, we tested whether HAT activity contributes to pol III transcriptional activation. H3-CoA-20-Tat is a cell-permeable peptide CoA conjugate that specifically inhibits PCAF and its close relative GCN5 (34). This treatment was found to compromise tRNA gene induction when added to MycER fibroblasts before OHT addition (Fig. 4D). No such effect was observed when cells were treated in parallel with a related, but inactive, peptide control. These data suggest that the stimulatory influence of GCN5 on pol III transcription involves its HAT activity.
Recruitment of TFIIIB and Pol III Is Stimulated by c-Myc and Trichos- tatin A (TSA). In addition to the modification of histones, c-Myc stimulates recruitment of TFIIH, P-TEFb, and mediator to the cyclin D2 promoter (13, 24). We therefore examined whether c-Myc also promotes assembly of pol III transcription factors. ChIP assays were carried out with MycER-transduced fibroblasts and antibodies specific for TFIIH, TFIIIC, and pol III (Fig. 5A). As expected, each of these proteins was detected at tRNA genes, but not at the cyclin D2 promoter or within the ARPP P0 coding region. As in Fig. 2A, pol III occupancy increased significantly after MycER induction, as did acetylation of histone H3 but not of histone H4. In addition, a marked increase in TFIIIB binding was observed. In contrast, TFIIIC occupancy remained unaltered when tested in parallel. These data suggest that c-Myc can specifically stimulate binding of pol III and TFIIIB to their target genes in vivo. This conclusion is reinforced by the experiments in Fig. 5B and SI Fig. 12, which show selective recruitment of TFIIIB and pol III to tRNA and 5S rRNA genes, but not to the ARPP P0 gene. Both the Brf1 and Bdp1 subunits of TFIIIB are recruited in response to c-Myc. In contrast, neither of the TFIIIC subunits examined shows any evidence of a change in occupancy. For both Brf1 and Bdp1, the increase in binding has occurred within 2 h of MycER induction, with no further increase detected after 4 h. This increase coincides with the recruitment of c-Myc and that of TTRRAP and GCN5 and the increased acetylation of histone H3. However, an increase in pol III assembly is not seen at the 2-h time point and only becomes apparent after 4 h. We conclude that activation of tRNA and 5S rRNA genes by c-Myc in vivo involves sequential assembly of TFIIIB followed by pol III.

To test whether elevated protein acetylation is sufficient to stimulate recruitment of TFIIIB and pol III, we treated cells with TSA. This drug inhibits histone deacetylases, thereby shifting the equilibria of acetylation reactions. Thus, Western blots show highly elevated acetylation of histones H3 and H4 in TSA-treated cells (SI Fig. 13A). ChIP assays confirm that this increase in acetylation applies to histones associated with pol III-transcribed genes (Fig. 5C). They also reveal a rapid increase in occupancy by TFIIIB and pol III. In contrast, little or no increase is seen in gene occupancy by TFIIIC. Therefore, TSA provokes the same selective recruitment of pol III factors as occurs after c-Myc induction. The response cannot be attributed to induced expression of these proteins, as Western blots show no change in the levels of representative subunits after TSA treatment (SI Fig. 13B). Instead, all of the data support a model in which HAT activity recruited via c-Myc stimulates assembly of active transcription complexes onto pol III-transcribed genes.

Discussion

This study presents a mechanistic analysis of how c-Myc stimulates tRNA and 5S rRNA synthesis in vivo. It identifies several ways in which activation resembles what has been reported for other classes of c-Myc targets. However, an unusual feature of pol III transcriptional induction is the selective increase in acetylation of histone H3, but not of histone H4. The clear increase in polymerase occupancy also contrasts with what has been observed at pol II-transcribed c-Myc targets. TTRRAP and GCN5 are detected at pol III-transcribed genes in vivo. RNAi-mediated depletion of either decreases tRNA gene expression. This result is unlikely to reflect off-target effects, as it was obtained with both an siRNA and a short hairpin that match different TTRRAP sequences, and the response to a GCN5 siRNA was independently confirmed by using a peptide inhibitor. We therefore conclude that TTRRAP and GCN5 are both involved in pol III regulation. This role appears to largely depend on c-Myc, as c-Myc null cells show markedly reduced TTRRAP occupancy of tRNA genes. However, a weak TTRRAP ChIP signal is retained in the knockout fibroblasts, albeit much diminished compared with the wild type; this result raises the possibility that TTRRAP or its associated proteins make contacts with the pol III machinery, although it is clear that any interaction is strongly promoted by c-Myc. The MBII region of c-Myc is responsible for its binding to TTRRAP and association with GCN5. MBII deletion prevents pol III induction by c-Myc. Furthermore, tRNA gene induction by c-Myc is attenuated when cells are treated with an inhibitor of GCN5 HAT activity. The data suggest a model in which c-Myc recruits a TTRRAP/GCN5 complex to pol III templates to stimulate their transcription. The positive effect of GCN5 on tRNA production is consistent with its well documented role in amino acid synthesis.

TTRRAP also forms a complex with the HAT PCAF, which is a homologue of GCN5 with very similar properties (11). We have not tested whether PCAF regulates pol III, because the siRNA and antibody reagents we used are specific for GCN5. However, the close similarity between these HATs makes it likely that PCAF also influences pol III transcription.
In contrast, the GCN5 and TIP60 complexes do not share common components, apart from TRRAP (12). TIP60 does not seem to be recruited to SS or tRNA genes after induction of c-Myc. This observation is consistent with the absence of increased H4 acetylation, as histone H4 is the preferred substrate of TIP60. In both of these respects, the mechanism of pol III activation by c-Myc differs from what is commonly seen for pol II, although there are known pol II exceptions (15, 21). TIP60 has also been detected at pol I-transcribed genes (33), as has H4 acetylation in response to c-Myc (29, 35). It is not clear why TIP60 is excluded from pol III templates, especially given the presence of TRRAP. Perhaps the TIP60-binding site on TRRAP is obscured by TFIIIB. It remains possible that TIP60 is present but not detected in our ChIPs, perhaps because of epitope masking.

Our data indicate that HAT activity stimulates recruitment of TFIIIB and pol III to their target genes, which may be explained by elevated histone acetylation, allowing increased access to DNA upstream of the transcription start site. Nevertheless, it is important to remember that HATs can acetylate proteins other than histones (36, 37). We have investigated whether c-Myc stimulates acetylation of the pol III machinery, but found no evidence that this is the case. However, we cannot exclude this mechanism as potentially contributing to the regulatory response.

Even in serum-starved fibroblasts expressing little or no c-Myc, tRNA and SS rRNA genes are marked by the presence of TFIIIC and acetylated histone H4. The latter observation may explain why HATs that target H4 may not be required for pol III stimulation by c-Myc, tRNA and 5S rRNA genes are marked by the presence of TFIIIC and acetylated histone H4 (38, 39). Perhaps its specificity is altered when bound to genes in vivo. Alternatively, a different HAT might associate with TFIIIC in cells. Whether or not TFIIIC has intrinsic HAT activity, it is noteworthy that it remains bound to genes under conditions where transcription is low. This finding is consistent with several previous studies in which TFIIIC was found to maintain high promoter occupancy, even in the face of unfavorable chromatin conditions (40). Indeed, it has been suggested that one function of TFIIIC is to resist nucleosomal silencing of pol III templates (41).

Recruitment of TFIIIB appears to be the stage at which c-Myc exerts its effect on pol III transcription. In this regard, it resembles many other regulatory factors, such as the retinoblas-toma protein (42). The increased gene occupancy by pol III in response to c-Myc may simply be a consequence of the elevated levels of promoter-bound TFIIIB, because TFIIIB is necessary and sufficient to bring pol III to its templates (43). However, this step might also be facilitated by c-Myc in some way. In contrast, high levels of pol II were found at the uninduced cad promoter irrespective of the presence of c-Myc (22). Indeed, no increase in pol II occupancy was seen at any of the six direct c-Myc targets examined by Bouchard et al. (24), including the cad and cyclin D2 promoters. TFIID and TFIIIB occupancy was also not enhanced (24). However, c-Myc induction did increase the occupancy of these promoters by P-TEFb, TFIIH, and Mediator (22, 24). This situation is reminiscent of the pol III system, where activation by c-Myc also involves recruitment of a basal factor (TFIIIB) onto promoters that are already marked by the presence of prebound machinery (TFIIIC).

Elevated levels of tRNA and SS rRNA can be expected to impact substantially on the protein synthetic capacity of a cell. Stimulation of pol III transcription may therefore make an important contribution to the biological efficacy of c-Myc, especially in terms of growth promotion. Both TRRAP and GCN5 are required for c-Myc to promote cell growth and transformation (10, 32), and we have shown here that they are also involved in pol III regulation. It is noteworthy that a recently discovered Myc target gene encodes Misu, an RNA methyltransferase that specifically methylates tRNA and is required for growth and proliferation of keratinocytes (44). Misu levels are abnormally elevated in a variety of human cancers, and RNAi-mediated knockdown of Misu reduces the growth of carcinoma xenografts in mice (44). Elevated production and processing of tRNA may therefore make a very significant contribution to Myc-induced tumorigenesis.

Methods

Cell Culture. HeLa and HEK293T cells were grown in DMEM (Cambrex Biosciences, East Rutherford, NJ) supplemented with 10% FBS (Sigma, St. Louis, MO), 2 mM l-glutamine, and 100 units/ml penicillin and streptomycin. Wild-type and c-Myc null fibroblasts were cultured as described (29). BALB/c 3T3 A31 fibroblasts transduced with empty pBabe vector or vector expressing MycER (pB-MYC-ER) were a generous gift from Carla Grandori (Fred Hutchinson Cancer Research Center, Seattle, WA); these cells were density-arrested and serum-starved before induction with 200 nM 4-OHT as described (29).

ChIP Assays. ChIP was performed as described (4). Immunoprecipitated DNA was quantified by PCR using published primers and amplification procedures (4). Antibodies were N-262 against c-Myc, H-75 against GCN5, C-19 against Max, M19 against TAFI48, FL-109 against TFIHA, C-18 against TFIIB, H-93 against TIP60, H-300 against TRRAP (Santa Cruz Biotechnologies, Santa Cruz, CA), 06-866 against acetylated histone H3, 06-599 against acetylated histone H4 (Upstate Biotechnology, Lake Placid, NY), MTBP-6 against TBP, 128 against Brf1, 4286 against TFIIC110, Ab4 against TFIIC220, 2663 against Bdp1, and 1900 against RPC155 (42, 45). Serial dilutions of chromatin were used to establish that PCRs were within a linear range.

Cell Extracts and Immunoblotting. Whole-cell extracts were prepared as described (46). For nuclear extracts, cells were washed twice with ice-cold PBS and scraped into hypotonic buffer (20 mM Hepes, pH 7.0/10 mM KCl/1 mM DTT/0.1% Triton X-100/20% glycerol/2 mM PMSF/5 μg/ml aprotinin/5 μg/ml leupeptin). The cell suspension was subjected to 10 strokes in a Dounce homogenizer and then centrifuged at 800 × g for 5 min. The supernatant (cytoplasmic fraction) was removed, and the pellet was resuspended in extraction buffer (20 mM Hepes, pH 7.0/420 mM NaCl/10 mM KCl/1 mM DTT/0.1% Triton X-100/20% glycerol/2 mM PMSF/5 μg/ml aprotinin/5 μg/ml leupeptin). The suspension was incubated end over end for 20 min at 4°C and then centrifuged at 16,000 × g for 10 min to generate the nuclear extract (supernatant).

Immunoblotting was performed as described (47) using antibodies F-7 against HA, C11 against actin, H-75 against GCN5, 9E10 against c-Myc, T-17 against TRRAP, C-20 against Oct-1 (Santa Cruz Biotechnologies); 06-866 and 06-599 against acetylated histone H3 or H4, respectively (Upstate Biotechnology); MTBP-6 against TBP, 128 against Brf1, 2663 against Bdp1, 1900 against RPC155, Ab4 against TFIIC220, 1898 against TFIIC90, and 3208 against TFIIC110 (45, 46).

RT-PCR Analysis. RT-PCR of ARPP P0 mRNA, 5S rRNA, and tRNA transcripts was performed as described (31, 48). RT-PCR of TRRAP mRNA used primers 5′-CAGGAAGTGAAACGCTTAGG-3′ and 5′-GTTTGGAGGTCT-GAAAGC-3′ to give a 252-bp product using the following cycling parameters: 95°C for 3 min, 25 cycles of 95°C for 1 min, 58°C for 30 s, 72°C for 1 min, 72°C for 5 min.

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RNAi. HeLa cells were transfected at 50% confluence to a final concentration of 100 nM siRNA oligonucleotides, delivered using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Medium was replaced after 5 h, and cells were harvested after an additional 72 h. The siRNAs used were TRRAP siRNA (h) sc-36746, GCNS siRNA (h) sc-37946, Oct-1 siRNA (h) sc-36119, all from Santa-Cruz Biotechnologies.