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Review

RNA polymerase III transcription—a battleground for tumour suppressors and oncogenes

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Abstract

This review provides a summary of the European Association for Cancer Research Award Lecture, presented at the ECCO12 meeting in Copenhagen in September 2003. It describes what we have learnt about the mechanisms responsible for deregulating RNA polymerase III transcription in transformed cells. A network has been discovered of unanticipated links to key tumour suppressors and oncogenes. Novel functions have been revealed for RB, p53 and c-Myc, that may help explain their profound biological effects.

Keywords: c-Myc; p53; Retinoblastoma; RNA polymerase III transcription; TFIIIB

1. Elevated levels of RNA polymerase III transcription are a feature of transformed cells

Eukaryotic cells use three RNA polymerases (pols) to transcribe the genes in their nuclei. Each is responsible for synthesising a different set of products: pol I synthesises the large rRNA and pol II synthesises the mRNA that is translated into proteins. Pol III is the largest RNA polymerase with the greatest number of subunits [1,2]. Its products are all short untranslated RNAs, including tRNA and 5S rRNA. This review will focus on how pol III transcriptional activity becomes deregulated in cancers.

A study published three decades ago reported that pol III is hyperactive in mice with myelomas [3]. A wide variety of transformed cell types was subsequently found to express abnormally high levels of pol III products, including lines transformed by DNA tumour viruses, RNA tumour viruses, or chemical carcinogens (for examples, see Refs. [4–17]). This activation is very general, but not universal, there being a few examples of transformed lines that do not display the characteristic increase in pol III transcript levels [4,6,18]. The abundance of pol III products varies substantially between different SV40-transformed lines and the highest levels correlate with progression to a more tumorigenic phenotype [4, 12]. Furthermore, cells transformed by temperature-sensitive mutants of the SV40 large T antigen activate pol III transcription within 30 min of transfer to the permissive temperature [19] and down-regulate pol III products when returned to the non-permissive temperature, whilst reverting to normal morphology and phenotype [4]. The relevance of such studies in culture has been validated for tumours *in situ*. For example, a pol III transcript called BC1, that is normally only detected in neurons, was found to be expressed in breast carcinomas, colonic adenocarcinomas and skin fibrosarcomas [20]. *In situ* hybridisation demonstrated the presence of BC1 RNA in the neoplastic cells, whereas it was absent from the surrounding tissues [20]. Similar studies have shown that BC200 RNA, the primate analogue of BC1, is expressed in many, but not all, primary human tumours [21]. Like BC1, BC200 RNA is found exclusively in the malignant cells and not in the adjacent normal tissue [21]. The same study looked at 7SL RNA, an essential pol III product that is involved in protein trafficking as part of the signal recognition particle; this revealed that 7SL RNA is overexpressed in every one of the 80 tumour samples analysed, relative to adjacent healthy tissue [21]. Half of these cases were breast carcinomas, but 19

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types of cancer were represented in the survey [21]. Levels of 7SL RNA were also found to be consistently elevated in ovarian tumours, along with tRNA and 5S rRNA [22]. Pol III hyperactivity therefore appears to be strongly associated with the transformed state.

2. Overexpression of pol III transcription factors in cancers

Although the link between pol III and cell transformation had been recognised for some time, the mechanistic basis of this phenomenon has taken longer to uncover. The most obvious way for tumours to increase pol III output is by raising the concentration of specific transcription factors. Two key proteins have been found to be targeted in this way. The first was TFIIIC2, a large DNA-binding factor that recognises promoter sequences directly and nucleates formation of the transcription complex on tRNA genes [1,2,23]. Studies with model systems revealed that TFIIIC2 is overexpressed at both the mRNA and protein levels following transformation of cell lines by SV40 or polyomavirus [12,16,17]. Much more importantly, the same phenomenon was detected in biopsy samples from cancer patients [22]. The DNA-binding activity of TFIIIC2 was measured in tissue from 9 patients with grade 2 or 3 ovarian carcinomas; in each case, the tumour sample had higher TFIIIC2 activity than adjacent healthy tissue from the same individual [22]. Reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that the tumours overexpress mRNAs encoding all five sub-units of TFIIIC2, whilst control mRNAs remained at normal levels [22]. This seems to be tied to transformation *per se*, rather than being a secondary response to accelerated proliferation, because TFIIIC2 levels are unaffected by growth factor availability or cell-cycle arrest [22,24]. These observations provided the first evidence that a pol III-specific factor is overproduced in cancers. Although the number of cases investigated was very small, the uniformity of response suggests that there is selective pressure to upregulate TFIIIC2 during ovarian tumorigenesis.

Once TFIIIC2 has bound to promoter DNA, it serves to recruit a three sub-unit factor called TFIIIB, which is responsible for positioning pol III at the transcription start site [1,2,23] (Fig. 1). In cell culture models, SV40 and polyomavirus can stimulate expression of one TFIIIB sub-unit [17], whereas another of its sub-units can be induced by hepatitis B virus [15]. In addition, we have recently found that TFIIIB sub-units are sometimes overexpressed in breast and cervical carcinomas (N.L. Daly, R.J. White, data not shown). Since TFIIIB and TFIIIC2 are dedicated exclusively to pol III transcription, these observations imply a specific drive to increase pol III output as the tumours develop.

3. Pol III transcription is suppressed by the retinoblastoma protein

Deregulation of TFIIIB in cancers is likely to be far more widespread than the instances detected so far in which the protein is overexpressed. This is because TFIIIB is subject to a complex network of regulatory controls, many of which become deregulated during oncogenic transformation. Perhaps the most important of these controls is exerted by the retinoblastoma tumour suppressor RB, which was found to possess a potent capacity to restrain pol III transcription [18]. Thus, transcription of pol III templates can be efficiently repressed by adding recombinant RB to *in vitro* systems or transfecting cells with RB expression vectors [18,25,26]. Conversely, synthesis of tRNA and 5S rRNA is markedly elevated in RB-knockout mice [18]. The ability of RB to elicit this response reflects its affinity for TFIIIB [25,27]. When bound by RB, TFIIIB is unable to interact with either TFIIIC2 or pol III [28]. As a consequence, RB can be a very potent inhibitor of pol III transcription.

RB is expressed almost ubiquitously in untransformed mammalian cells, where it serves to ensure that growth and proliferation do not proceed under inappropriate conditions [29,30]. It achieves this by binding and regulating a wide range of transcription factors, including the pol I-specific factor UBF, the pol II-specific factor E2F and the pol III-specific factor TFIIIB, each of which influence a cell's capacity to progress through the cell cycle or grow [30]. When conditions are propitious, in terms of nutrients and mitogens, RB is phosphorylated at multiple sites by cyclin D- and E-dependent

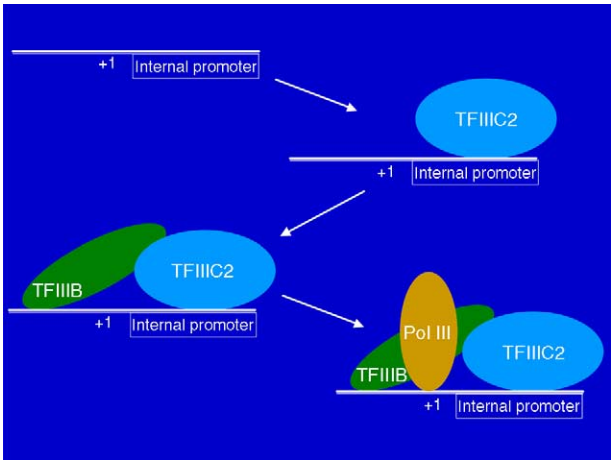

Fig. 1. Schematic illustration of the assembly of a transcription complex on a typical RNA polymerase III (pol III) template, such as a tRNA gene. These genes have internal promoters, located downstream of the transcription start site (+1) within the transcribed region. This is recognised by the large DNA-binding factor TFIIIC2. TFIIIC2 recruits TFIIIB to the promoter by protein/protein interactions. TFIIIB, in turn, recruits pol III itself and positions it over the start site so that transcription can commence.

kinases [30]. In its hyperphosphorylated form, RB dissociates from its transcription factor targets, releasing them from its restraining influence. In this way, RB serves to ensure that various key genes are only transcribed at maximal rates when their products are required for growth and/or cell-cycle progression.

4. Disruption of the RB pathway in cancers may deregulate pol III transcription

Because of its restraining influence, RB serves as a barrier against tumour development. To overcome this barrier, RB function must be compromised and this is thought to be a necessary step in the transformation process. The most common way that this is achieved is through constitutive hyperphosphorylation of RB, so that it loses its ability to bind and regulate transcription factors. Many tumours overexpress cyclin D in order to activate its associated kinases, cdk4 and cdk6, and thereby phosphorylate RB constitutively. For example, the gene for cyclin D1 is amplified in at least 15% of primary breast cancers and an even greater proportion of squamous cell carcinomas of the neck, head, oesophagus and lung [31,32]. Furthermore, cyclin D1 RNA and protein is overexpressed in 30–40% of primary breast tumours, suggesting that gene amplification is not the only mechanism contributing to increased levels of the product [32]. In some parathyroid adenomas and B cell lymphomas, chromosomal translocations cause overproduction of cyclin D1 [31,32]. When Epstein–Barr virus immortalises B-lymphocytes, cyclin D2 becomes activated [33]. The gene for cdk4 is amplified in many glioblastomas and some gliomas [29]. In addition to these diverse situations in which cyclins or their associated kinases are activated directly, many other cancers lose the function of p16, an important repressor of the cyclin D-dependent kinases [29,31,34]. For example, the gene for p16 is frequently deleted or mutated in pancreatic, oesophageal and bladder carcinomas, as well as many familial melanomas [34–36]. It is also common for transcription of wild-type p16 alleles to be silenced through promoter methylation [35]. Gene deletion, mutation or methylation together deprive approximately 75% of pancreatic tumours of wild-type p16 expression [35]. Thus, the cyclin D-dependent kinases become abnormally active in a broad spectrum of cancers through a variety of mechanisms. In effect, this switches off RB, causing it to dissociate from many of its targets. TFIIIB only binds to hypophosphorylated RB and is released from repression once the latter is inactivated by cyclin D-dependent kinases [24]. Accordingly, pol III transcription can be stimulated by overexpression of cyclin D and cdk4, or by depletion of p16 [24].

A significant fraction of tumours have no need to activate cyclin D-dependent kinases because they carry mutations in RB itself. These include carcinomas of the breast, prostate and bladder, as well as osteosarcomas. The most striking examples are small cell lung carcinomas, nearly all of which carry mutations in the *RB1* gene [37]. As well as these sporadic cancers, in which the mutations arise during tumorigenesis, some individuals inherit a mutant form of the gene, which results in an approximate 90% chance of developing retinoblastoma at an early age [38,39]. Most (98%) mutations in the *RB1* gene will affect a region of its protein product called the large pocket, which spans residues 393–928 [39,40]. This region of RB is necessary and sufficient for its interaction with TFIIIB, as well as many other targets [25,27]. Accordingly, mutations in the pocket can prevent RB from repressing pol III transcription [18,25,41,42]. These include some very subtle changes, including missense mutations from retinoblastoma or small-cell lung carcinoma patients [18,41]. In contrast, a low-risk substitution mutant associated with benign retinomas was found to retain a significant capacity to repress pol III transcription, albeit incomplete [42].

As well as mutation and hyperphosphorylation, RB can also be inactivated through the binding of viral oncoproteins [43]. The most important of these is the E7 product of human papillomaviruses (HPVs), which play an aetiological role in most cervical neoplasias [44,45]. HPV is also associated with approximately 25% of oropharyngeal cancers [44]. The E7 oncoprotein can bind to RB and transform established cell lines [46,47]. E7 from the high-risk viruses, HPV-16 and -18, has a higher affinity for RB than E7 from the less oncogenic strains HPV-6 and -18 [47,48]. However, substitutions in HPV-6 E7 that cause a substantial increase in affinity for RB also produce a concomitant gain in transforming activity [48,49]. Pol III transcription can be stimulated strongly by transfecting RB-positive cells with HPV-16 E7 [16,50]. E7 substitutions that prevent RB binding, also ablate its ability to stimulate pol III output [16,50].

The transforming proteins of several other DNA tumour viruses can also bind the RB pocket and neutralise its function [43]. This property is shown by the large T antigens of simian virus 40 (SV40) and polyomavirus [51–54] and the E1A protein of adenovirus [55,56]. The regions of these oncoproteins that are necessary for RB binding are also required for their transforming properties [51–53,56]. By binding to RB, these viral proteins can interfere with its normal cellular functions and thereby mimick the effects of the *RB1* mutations that occur in many tumours. As one might predict, RB-mediated repression of TFIIIB can be overcome by E1A and the large T antigens of SV40 and polyomavirus, thereby releasing pol III transcription from a major restraining influence [16–18].

Thus, derepression of TFIIIB would seem to be a consequence of each of the mechanisms that compromise RB function in tumours—hyperphosphorylation,

genetic mutation, or sequestration by transforming proteins (Fig. 2). Since one or other of these mechanisms is believed to function in most if not all cancers, the data predict that release from RB-mediated repression may provide a universal mechanism to deregulate pol III transcription in tumours.

5. p53 can also suppress pol III transcription, a function that may be lost in tumours

In addition to RB, p53 is also involved in restraining pol III output, perhaps providing a fail-safe mechanism that backs up RB when cells are challenged by potentially oncogenic stresses. The fact that the pol III system is targeted by these two cardinal tumour suppressors provides a clear indication of its importance. Indeed, there are good reasons to consider that restraining rRNA and tRNA synthesis may provide an important component of the growth control function of these tumour suppressors [57–59]. Like RB, p53 inhibits pol III transcription by binding to TFIIIB [60–62]. Once bound by p53, TFIIIB loses its ability to interact with TFIIIC2 [62]. Since this interaction is required to recruit TFIIIB to promoters (Fig. 1), p53 induction is accompanied by a marked decrease in TFIIIB occupancy at pol III-transcribed genes [62]. For example, when cells are treated with a DNA-damaging drug that induces p53, the presence of TFIIIB at tRNA genes decreases markedly, as does their transcription [62]. Conversely, tRNA synthesis is unusually high in p53-knockout mice, in keeping with a substantial increase in the fraction of promoters that are occupied by TFIIIB [61,62].

At least half of human tumours carry mutations in p53, most of which map to its central domain [63,64].

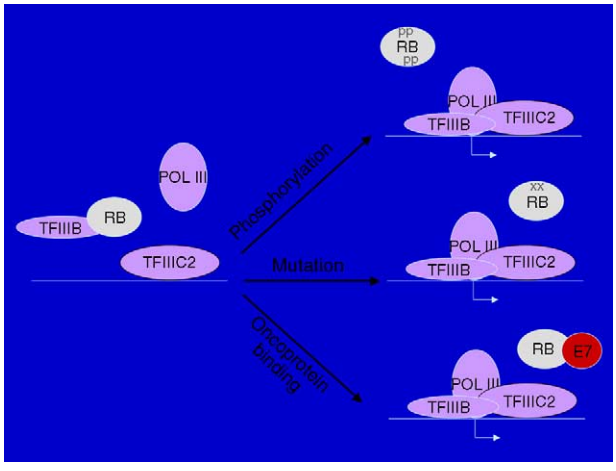

Fig. 2. RB binds to TFIIIB and prevents its interactions with TFIIIC2 and RNA polymerase III (pol III), thereby blocking transcription complex assembly. This function can be lost in cancers through one of three mechanisms—constitutive phosphorylation (pp) of RB, mutation (xx) of RB, or the binding to RB of oncogene products such as human papillomavirus (HPV)-16 E7.

Since this domain is required for p53 to repress TFIIIB [65], pol III transcription may be derepressed in cancers with such mutations. Indeed, several tumour-derived substitutions in p53 have been shown to compromise its ability to repress a pol III reporter [66]. Individuals who inherit mutant forms of p53 can suffer from Li–Fraumeni syndrome, a familial cancer predisposition [67]. Approximately half the members of Li–Fraumeni families who inherit a mutated p53 allele develop cancer before they reach the age of 30 years [67]. Primary cells from such patients frequently display elevated pol III transcriptional activity, although exceptions also occur [66]. Apart from genetic mutations, p53 function can be lost in some tumours through the action of viral or cellular oncogenes. For example, the E6 oncoprotein of HPV promotes p53 degradation in most cervical carcinomas [44,45]. Similarly, p53 is degraded after binding to the cellular oncoprotein hdm2, which is overexpressed in a range of cancer types, including osteosarcomas and soft-tissue tumours [68]. Accordingly, both E6 and hdm2 can stimulate pol III transcription by relieving TFIIIB from p53-mediated repression [66].

6. TFIIIB is targeted directly by several oncogenic factors

In view of the frequency with which tumours lose the function of p53 and/or RB, the resultant derepression of TFIIIB might seem to be sufficient to allow elevated pol III transcription in most if not all cancers. However, it is now evident that TFIIIB is also directly activated by certain oncogenic proteins. One of these is the Tax product of human T cell leukaemia virus I [14]. Another is the kinase CK2, which binds and phosphorylates TFIIIB and facilitates its recruitment by TFIIIC2 [69,70]. CK2 is oncogenic in transgenic mice and is abnormally active in some human cancers [71–74]. In such situations, its hyperactivity might contribute to an increase in pol III transcription. TFIIIB is also bound, phosphorylated and activated by the MAP kinase Erk [75]. The MAP kinase cascade leading to Erk is induced in approximately 30% of tumours [76]. This can occur through overexpression of the EGF receptor or ERBB2 (also known as HER2/neu), as is common in breast and ovarian carcinomas, or mutation of the kinase BRAF, a feature of most melanomas [76]. However, the most frequent cause is the mutational activation of the ubiquitous oncoprotein Ras, which is found in 20% of all tumours, including 90% of pancreatic cancers [76]. TFIIIB is likely to be hyperphosphorylated and hyperactive in such cases.

The oncogene product c-Myc also binds to TFIIIB and stimulates pol III transcription [77]. Indeed, chromatin immunoprecipitation has revealed the presence of c-Myc at tRNA and 5S rRNA genes in several cell types

[42,77]. Depletion of c-Myc by RNA interference has shown that it helps drive pol III transcription in carcinoma cells [42]. Conversely, its overexpression can induce a rapid and dramatic increase in tRNA and 5S rRNA synthesis [77]. Deregulation of c-Myc is found in a wide range of malignancies, including virtually every case of Burkitt's lymphoma [78,79]. In all, it has been estimated that c-Myc may contribute to one-seventh of United States (US) cancer deaths [78].

From these studies, it has become clear that the pol III-specific transcription factor TFIIIB lies within a complex network of regulatory influences (Fig. 3). It is targeted by two of the cell's key tumour suppressors, RB and p53, which act to restrain its function and thereby limit the output of pol III. This effect is reinforced by the p16 tumour suppressor, that helps to maintain RB in the underphosphorylated state in which it can bind to TFIIIB. However, a variety of oncogenic aberrations can subvert this control. These include overexpression of cyclin D, hdm2, or viral products such as E6 and E7, as well as mutations in the tumour suppressors themselves. In addition, TFIIIB is activated directly by the kinases CK2 and Erk and the oncoproteins Tax and c-Myc. Activation of Ras can also stimulate TFIIIB function, both by switching on Erk and by inducing cyclin D expression. So TFIIIB may be viewed as something of a battleground, which is contested by tumour suppressors, that try to restrict its activity to appropriate levels, whereas batteries of oncogenic influences attempt to subvert that control. The outcome of this struggle will have profound consequences for the balance of nuclear activity.

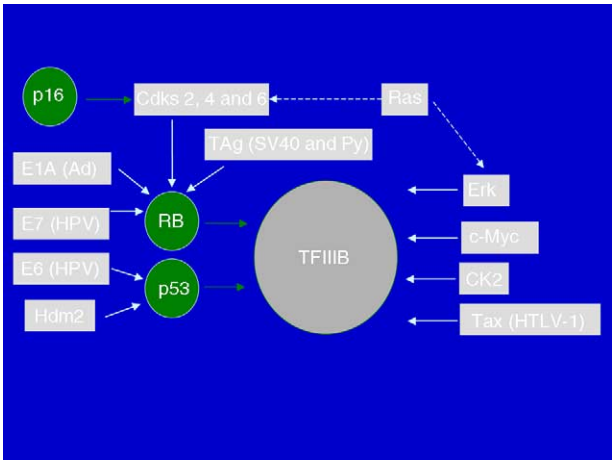

Fig. 3. Tumour suppressors (spheres) and oncogene products (rectangles) battle to control TFIIIB activity. RB and p53 act directly on TFIIIB to restrain its function. This control can be subverted by the cellular oncoprotein Hdm2, the E6 and E7 products of high-risk human papillomaviruses, adenoviral E1A, the large T-antigens of SV40 and polyomavirus, or the cyclin D- and E-dependent kinases cdk2, cdk4 and cdk6. The tumour suppressor p16 helps maintain TFIIIB repression by inhibiting RB phosphorylation. TFIIIB is activated directly by c-Myc, the Tax product of human T-cell leukaemia virus I, and the kinases CK2 and Erk. Oncogenic mutations in Ras will activate Erk and induce cyclin D production, leading to further activation of TFIIIB.

7. Rapid growth requires high rates of pol III transcription

To maintain a constant size, a cell must duplicate its components prior to division. Since 80–90% of a cell's mass is protein, a high rate of protein synthesis is a prerequisite of rapid growth [80]. Indeed, the growth rate is directly proportional to the rate at which protein accumulates [81]. Furthermore, a 50% reduction in translational output is sufficient to cause cells to withdraw from cycle and quiesce [82,83]. Cells cannot enter S phase or duplicate their chromosomes until sufficient protein has accumulated [84,85]. Since the availability of tRNA and rRNA is a principle determinant of protein synthetic capacity, high rates of pol III transcription are essential for cells to maintain rapid growth. Thus, protein synthesis soon reaches a limit in fibroblasts experiencing a tRNA deficit, whilst a 2-fold reduction in the level of a tRNA can cause a 3-fold increase in cell doubling time [86,87]. These facts seem sufficient to explain why tumours select for high pol III activity—it may be necessary to provide the potential for sustained or accelerated growth.

8. Summary

The pol III-specific transcription factor TFIIIB is a major determinant of biosynthetic capacity. It is targeted by many key regulatory proteins and may therefore provide a major control point in dictating cellular activity. It is bound and repressed by RB and p53 in untransformed cells. Repression of pol III transcription through TFIIIB may contribute to the growth-restraining functions of these two cardinal tumour suppressors. As p53 and/or RB become inactivated during oncogenic transformation, TFIIIB will be freed from restraint. Its consequent deregulation will often be aggravated by oncogene products such as c-Myc, which can stimulate its activity directly. The network of regulators that act on TFIIIB may therefore shift dramatically as cells transform. In addition, some tumours reinforce these effects by raising production of pol III transcription factors. For example, ovarian carcinomas overexpress TFIIIC2, which recruits TFIIIB to its target genes. The data suggest that cancer cells are very keen to achieve a high pol III output, which is probably a prerequisite for accelerated growth. It remains to be determined if the pol III system can provide a useful target for therapeutic intervention.

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