MECHANISMS OF RNA POLYMERASE III TRANSCRIPTION IN HUMAN CELLS

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If DNA is often referred to as the molecule of life, then gene expression, which leads, ultimately, to execution of the instructions embedded in the DNA, is certainly the process of life. Basal cellular metabolism, cell division, and the intricate processes of cellular differentiation and adaptation to external conditions all depend on the controlled expression of specific genes, starting with the process of transcription. Perhaps not surprisingly, then, the complexity of the machineries involved in gene expression and in particular in transcription increases with the complexity of organisms, and in mammalian genomes, a sizeable percentage of the proteome seems to be involved in the control of transcription (see for example [Waterston et al., 2002]).

Eukaryotes possess three main nuclear DNA-dependent RNA polymerases, RNA polymerases (pol) I, II, and III, each dedicated to the transcription of specific classes of genes. Pol I and III synthesize RNA molecules required for fundamental cellular processes such as RNA processing and protein synthesis; thus, pol I transcribes the 45S gene, generating the 28S, 18S, and 5.5S ribosomal RNAs, and pol III synthesizes structural and catalytic RNA components of the protein synthesis and RNA processing machineries such as, for example, 5S RNA, the tRNAs, and the spliceosomal U6 small nuclear RNA (snRNA), as well as short RNAs of unknown function. Pol III also transcribes micro RNAs as part of some repetitive sequences such as Alu sequences (Borchert et al., 2006). The main task of pol II is the transcription of protein-encoding genes, although it also transcribes some small nuclear RNA genes such as, for example, the U1 and U2 snRNA genes, involved in mRNA splicing. Among the three transcription machineries, the pol II machinery is the most complex, reflecting the highly varied protein constellations present in different cells at different times, and the need for intricate regulation of such protein

patterns, whereas the pol I machinery, which transcribes only one type of gene, is probably the least complex.

The largest effort in the field of transcription has gone toward the study of mechanisms of transcription by pol II. However, there is a growing interest in deciphering how pol I and III transcription is regulated, as this is key to understanding questions as fundamental as how can a cell adapt its protein synthesis capability to its changing needs. In this review, I will summarize what we know about the pol III basal transcription machinery in mammalian cells, and about the regulation of this machinery.

Three main types of pol III promoters

Pol I, being dedicated to transcription of the highly repeated 45S genes, needs to recognize, together with its transcription factors, only one promoter structure. Pol II is at the other extreme; it recognizes promoters that often contain separable core and regulatory regions and that are enormously varied, especially in their regulatory regions. Pol III recognizes a more limited number of promoter structures and thus is, in this respect, intermediate between pol I and pol II. The pol III promoters have been classified into three main types, of which the first two are gene-internal and generally TATA-less, and the third is gene-external and contains a TATA box (see [Schramm and Hernandez, 2002] for a review). These promoters are illustrated in Figure 1. Type 1 and 2 promoters are found in the 5S rRNA genes and in tRNA genes, respectively. The Xenopus Laevis 5S gene promoter, which often serves as the model type 1 promoter, is called internal control region (ICR) and consists of an A box, an intermediate element (IE), and a C box that is conserved in the 5S promoters of different species (Bogenhagen et al., 1980, Sakonju et al., 1980). Most tRNA promoters as well as the Adenovirus VA1 promoter, often used as a model type 2 pol III promoter because of its exceptional strength, consist of an A box and a B box, which can be spaced differently in different genes (Fowlkes and Shenk, 1980, Galli et al., 1981, Hofstetter et al., 1981, Sharp et al., 1981). Type 3 promoters are not found in yeast, and are exemplified by vertebrate

U6 snRNA promoters (Das et al., 1988, Krol et al., 1987, Kunkel and Pederson, 1988). The human U6 promoter consists of a TATA box and a proximal sequence element (PSE), both of which are required for basal transcription in vitro. In addition, the promoter contains a distal sequence element (DSE), which enhances transcription from the basal promoter. The type 3 promoters have the particularity of being very similar to promoters such as the human U1 and U2 promoters, which are recognized by pol II and consist of a PSE and a DSE without a TATA box (see [Hernandez, 2001] for a review). Finally, a number of pol III promoters are mixtures of elements constituting the basic type 1, 2, and 3 promoters. For example, the selenocysteine tRNA promoter from Xenopus laevis combines DSE, PSE and TATA box in the 5' flanking region with a gene-internal B box (Carbon and Krol, 1991).

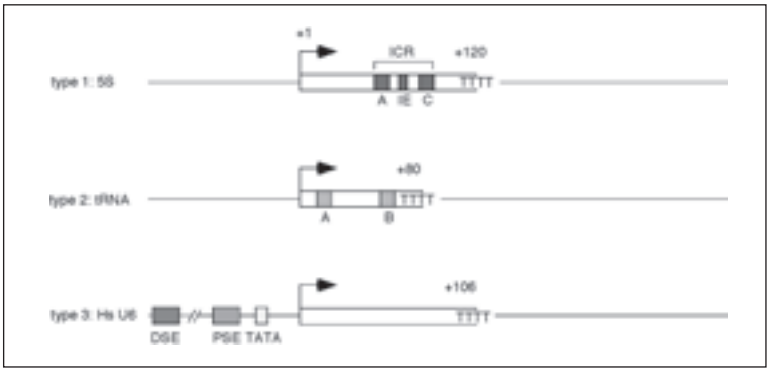

Figure 1
Structure of the three types of pol III promoters.

The various types of pol III promoters recruit different transcription factors

Like other RNA polymerases, pol III cannot recognize its target promoters without the help of accessory factors that bind to promoter elements and in turn recruit other accessory factors and the polymerase. As illustrated in Figure 2, for type 1 promoters, the ICR is recognized by a zinc finger protein referred to as TFIIIA (Engelke et al., 1980, Sakonju et al., 1981). Formation of the TFIIIA-DNA complex then

allows the binding of TFIIIC (Lassar et al., 1983), and then that of Brf1-TFIIIB (Schramm et al., 2000, Teichmann et al., 2000). Once Brf1-TFIIIB is bound, pol III can be recruited to the promoter. For type 2 promoters, TFIIIA is not needed to assemble the preinitiation complex. Instead, TFIIIC binds directly to the A and B boxes and recruit Brf1-TFIIIB and then pol III. In the case of type 3 promoters, a different set of transcription factors is needed. The PSE is recognized by a factor we call the snRNA activating protein complex ($SNAP_c$), also known as PTF, and the TATA box is recognized by Brf2-TFIIIB (see (Geiduschek and Kassavetis, 2001, Hernandez, 2001, Schramm and Hernandez, 2002) for reviews). This then allows the recruitment of the polymerase. Thus, for all pol III promoters, the assembly of the preinitiation complex leads to recruitment of either Brf1- or Brf2-TFIIIB, which then allows pol III recruitment.

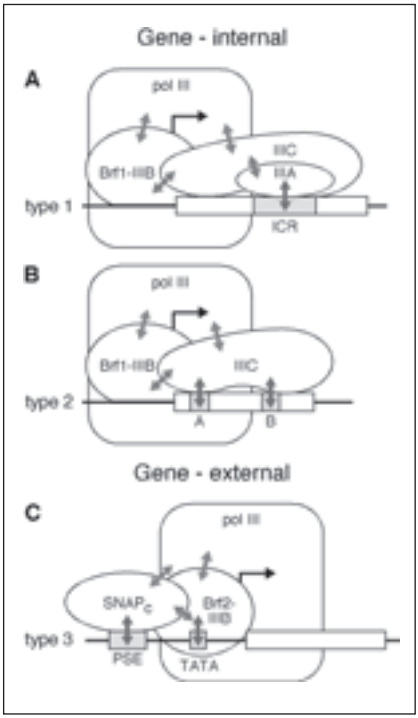

Figure 2
Recruitment of pol III to types 1, 2, and 3 promoters. The arrows indicate either DNA-protein or protein-protein contacts. In the case of type 3 promoters, the contacts between pol III and $SNAP_c$ or Brf2-TFIIIB are putative.

Transcription factors IIIA and IIIC

In the last twenty years or so, a large effort has been devoted to the characterization of the transcription factors mentioned above, which were, originally, uncharacterized activities in different chromatographic fractions. This led to the cloning of cDNAs encoding all of these factors, and thus to a detailed description of the pol III basal transcription machinery. Indeed, for some yeast and human pol III promoters, we can now reconstitute basal pol III transcription with entirely recombinant transcription factors and highly purified pol III. TFIIIA was the first eukaryotic transcription factor to be cloned (Ginsberg et al., 1984), and its characterization then led to the concept of zinc finger proteins (Miller et al., 1985). It indeed contains nine C_2H_2 zinc fingers. In yeast, its only essential role is in the transcription of the 5S RNA gene, because yeast strains engineered to express 5S RNA from a type 2 promoter and lacking TFIIIA are viable (Camier et al., 1995). TFIIIC is a large complex composed of six types of subunits, some of which are only moderately conserved from yeast to human cells (Deprez et al., 1999, Dumay-Odelot et al., 2007, Hsieh et al., 1999a, Hsieh et al., 1999b, Kundu et al., 1999, L'Etoile et al., 1994, Lagna et al., 1994, Lefebvre et al., 1992, Manaud et al., 1998, Marck et al., 1993, Swanson et al., 1991, Willis et al., 1989). A functional yeast TFIIIC complex has recently been reconstituted from recombinant proteins expressed in insect cells with baculovirus vectors (Ducrot et al., 2006).

$SNAP_c$

$SNAP_c$ is found only in metazoans. In mammalian cells, it is composed of five types of subunits, SNAP190, SNAP50, SNAP45, SNAP43, and SNAP19, all of which have been cloned (Bai et al., 1996, Henry et al., 1996, Henry et al., 1998, Henry et al., 1995, Sadowski et al., 1996, Wong et al., 1998, Yoon and Roeder, 1996), and a recombinant complex can be synthesized in insect cells from baculovirus vectors (Henry et al., 1998, Mittal et al., 1999).

Brf1-TFIIIB and Brf2-TFIIIB

In yeast, there is only one TFIIIB activity, which is minimally composed of three polypeptides: the TATA box binding protein TBP, the TFIIB-related factor Brf1, and the SANT domain protein Bdp1 (see

Geiduschek and Kassavetis, 2001, Schramm and Hernandez, 2002, for reviews). In human cells, however, there are two TFIIB-related factors, Brf1 and Brf2, and consequently two TFIIIB activities: Brf1-TFIIIB contains human TBP, Bdp1, and Brf1, whereas Brf2-TFIIIB contains TBP, Bdp1, and Brf2. As described above, Brf1-TFIIIB is recruited to type 1 and 2 promoters, and Brf2 to type 3 promoters(Mital et al., 1996, Schramm et al., 2000, Teichmann et al., 2000).

TBP, Brf1, Brf2, and Bdp1 have all been cloned and can be expressed in E. coli (Mital et al., 1996, Schramm et al., 2000, Teichmann et al., 2000, Wang and Roeder, 1995). Brf1 and Brf2 are of particular interest. As their name indicates, they are related to TFIIB, containing, like TFIIB, a zinc-binding N-terminal domain and a core domain consisting of two repeats. Over most of these regions, the three proteins are approximately 20% identical (Schramm et al., 2000, Teichmann et al., 2000). Brf1 and Brf2 contain, in addition, C-terminal extensions that are absent in TFIIB and that appear unrelated to each other.

At pol II promoters, TFIIB bridges pol II with the promoter-bound preinitiation complex, whereas Brf1 and Brf2 are involved, as part of Brf1-TFIIIB and Brf2-TFIIIB, in the establishment of a pol III transcription initiation complex and in the recruitment of pol III. How do these related factors establish pol II versus pol III specificity? In yeast Brf1, the C-terminal extension is known to interact with the TBP/TATA box complex and to contribute to the recruitment of Bdp1, which presumably leads, in turn, to the recruitment of pol III. Significantly, a structure-function analysis of human Brf2 suggests that the C-terminal extension of Brf2 is required for efficient association of the protein with U6 promoter-bound TBP and $SNAP_c$, and for efficient recruitment of Bdp1 (Saxena et al., 2005). Close inspection of the C-terminal domain of Brf2 reveals a short region that can be aligned with the Brf1 region required for efficient binding to the TBP/TATA box complex. Thus, a common feature among TFIIB family members involved in pol III transcription appears to be the use of their C-terminal extension to associate efficiently with a TBP/TATA box complex and to recruit Bdp1. This in turn suggests that the C-terminal extensions in Brf1 and Brf2 are key to specific recruitment of pol III over pol II (Saxena et al., 2005).

Bdp1

Bdp1 was first cloned in yeast cells by biochemical methods(Kassavetis et al., 1995, Roberts et al., 1996, Rüth et al., 1996), and shown to contain a domain related to a Myb repeat. This domain was identified by comparison of the SWI-SNF and ADA complexes, the transcriptional co-repressor N-Cor, and yeast TFIIIB, and was therefore named the SANT domain (Aasland et al., 1996). The SANT domain of the yeast protein is required for function (Kassavetis et al., 1998), and so human Bdp1 cDNAs were isolated through a combination of database searches for sequences similar to the yeast Bdp1 SANT domain and library screening (Schramm et al., 2000). This led to the identification of Bdp1 splicing variant 1 (Bdp1_v1), which is highly related to the yeast protein within the SANT domain (43% identities) as well as in regions both immediately upstream (21% identities) and downstream (17% identities). Outside of these regions, the two proteins are not conserved, and the human protein differs from the yeast protein by a striking C-terminal extension containing a number of repeats. Other Bdp1 cDNAs corresponding to alternatively spliced mRNAs potentially encode a very large protein in which the last few amino acids of Bdp1_v1 are replaced by a 901 aa extension (Bdp1_v2), and a 725 aa protein (Bdp1_v3) corresponding to Bdp1_v1 sequences up to aa 684, followed by a divergent 47 aa extension (Kelter et al., 2000). Whether all of these alternatively spliced forms of human Bdp1 are involved in pol III transcription in vivo is currently not clear.

Regulation of pol III transcription

Pol III synthesizes RNAs that are often abundant and stable. As described above, these RNA molecules then participate in the processing of other RNA molecules, in protein synthesis, and in other essential metabolic reactions. Thus, in cells that are actively dividing, pol III activity is high such that an entire complement of pol III products can be produced within one cell generation. Indeed, cancer cells are characterized by increased pol III transcription activity. Similarly, in growing cells, pol III activity is high to meet the increased demand for such molecules. On the other hand, in resting cells, pol III activity is mostly

limited to the replacement of the few pol III products that decay over time. Thus, pol III transcription is highly regulated with cell proliferation and growth. Moreover, pol III transcription is very rapidly inhibited after certain stresses such as DNA damage and serum starvation. Accordingly, there is a high interest in understanding the regulation of pol III transcription, the role of such regulation in continued cell division, and the coordination of such regulation with that of pol I transcription and pol II transcription of certain genes involved in, for example, ribosome biogenesis.

Pol III transcription is regulated by at least two very general mechanisms, the first acting through regulatory promoter sequences and the second through the basal transcription machinery. Indeed, although in mammalian cells, the core pol III promoters, which correspond to one of the three types of promoters described in Figure 1 or, in some cases, to a mixture of elements from the different types, are generally thought to direct most of the total transcription activity, there are several examples where flanking sequences are known to modulate the activity of the core promoter (see for example [Howe and Shu, 1989]), and closer examination of natural cellular pol III promoters in human cells would most likely reveal that this is a quite common occurrence. Thus, part of the regulation of pol III transcription is exerted through factors binding to such modulatory sequences, which then activate or, conceivably, repress, pol III transcription. In the second mechanism, regulators act directly on the basal transcription factors described above. Since the basal transcription factors are used by entire classes of pol III promoters, such mechanisms can potentially repress a large collection of pol III genes in a concerted manner.

Transcription activation through regulatory promoters sequences: the human U6 promoter

Perhaps the best studied example of regulation through promoter regulatory sequences is that of the type 3 human U6 snRNA promoter. As described above in Figure 1, type 3 promoters contain a distal sequence element (DSE). The DSE can be composed of various protein binding

sites, but one of them is almost always an octamer sequence that recruits the POU domain transcription factor Oct-1, and the other an SPH (for "*Sph1* postoctamer homology") element that recruits the zinc finger protein Staf (ZNF143) (see [Hernandez, 2001] for a review). How do these factors activate transcription?

In the case of Oct-1, we know that both the activation domain of the protein as well as the DNA binding domain contribute to transcription activation. The mechanism by which the Oct-1 activation domain does so is not known, but the Oct-1 DNA binding domain binds cooperatively with $SNAP_c$ to the U6 promoter and so helps $SNAP_c$ recruitment. The DNA binding domain of Oct-1 is a POU domain, a bipartite DNA binding domain consisting of two helix-turn-helix-containing DNA-binding structures: an amino-terminal POU-specific (POU_S) domain and a carboxy-terminal POU-homeo (POU_H) domain, joined by a flexible linker (Herr and Cleary, 1995). Cooperative binding results from a direct protein-protein contact between the Oct-1 POU_S domain and the largest subunit of $SNAP_c$, contact that is mediated by a nucleosome positioned between the PSE and the DSE in the U6 promoter (Ford et al., 1998, Zhao et al., 2001).

Oct-1 is not capable to bind to pre-assembled chromatin in vitro, suggesting that activation of U6 snRNA gene transcription in vivo requires the prior binding of another factor which can then either recruit Oct-1 directly or somehow modify chromatin to make it accessible to Oct-1. A strong candidate for such a factor is Staf (ZNF143). Indeed, Staf cannot only activate U6 transcription from a chromatin template in vitro like Oct-1, it is in addition capable of binding to preassembled chromatin (Yuan et al., 2007). Moreover, Staf associates with a number of factors that are involved or are likely to be involved in chromatin modification. One of these, chromodomain-helicase-DNA binding protein 8 (CHD8), resides on the human U6- as well as on some pol II promoters in vivo, and contributes to efficient U6 transcription. Thus, pol III transcription is likely to use at least some of the same factors as those used for chromatin remodeling at pol II promoters.

Transcription regulation through the basal pol III transcription machinery

A number of factors have been shown to regulate pol III transcription through the general pol III transcription machinery. These include Rb, c-Myc, PNRC, p53, Maf1, CK2, and the list is growing! All of these factors also regulate transcription of some pol II genes and in some cases of the pol I rRNA genes.

Pol III transcription is regulated with the cell cycle. When Balb/C 3T3 fibroblasts are arrested in G0 by serum deprivation, pol III transcription from class 2 promoters is greatly reduced. It is reactivated shortly after serum addition, well before the G1/S boundary, through a pathway involving the mitogen-activated protein kinase ERK, which binds to and phosphorylates Brf1 (Felton-Edkins et al., 2003). In cycling cells, pol III transcription is low in G1 and high in the S and G2 phases (Scott et al., 2001, White et al., 1995). Consistent with this observation, type 2 pol III promoters are repressed by Rb as well as p107 and p130, which bind to an undefined Brf1-TFIIIB subunit and prevent Brf1-TFIIIB association with promoters (Larminie et al., 1997, Sutcliffe et al., 2000, Sutcliffe et al., 1999, White et al., 1996). Transcription from the type 3 U6 promoter is also inhibited by Rb, which in this case associates with $SNAP_c$ through two of the $SNAP_c$ subunits (Hirsch et al., 2000, Hirsch et al., 2004). Interestingly, Rb repression of U6 transcription requires the Rb A/B pocket as well as the C domain, which is not required for repression of pol II transcription but which is part of the domain required for growth suppression. This suggests that repression of pol III transcription is an important component of growth suppression (Hirsch et al., 2004). During mitosis, pol III transcription is repressed by the cdc2/cyclin B kinase, either through direct phosphorylation of a Brf1-TFIIIB subunit or by activation of secondary kinases, which in turn phosphorylate a Brf1-TFIIIB subunit (Gottesfeld et al., 1994, Leresche et al., 1996). One such secondary kinase is CK2, which can inactivate Bdp1 by direct phosphorylation (Hu et al., 2004). CK2 can also activate transcription, however, through phosphorylation of pol III itself or a pol III-associated factor (Hu et al., 2003). Thus, CK2 has both positive and negative roles in pol III transcription.

A number of positive regulators of pol III transcription have been described. c-Myc binds to Brf1-TFIIIB, activates class 1 and 2 pol III promoters, and can be found on these promoters in vivo by chromatin immunoprecipitation experiments (Gomez-Roman et al., 2003). Similarly, PNRC, a nuclear receptor coactivator that regulates a wide range of pol II-transcribed genes, associates with the RPC30 subunit of pol III and can be localized on pol III promoters by chromatin immunoprecipitation experiments. Further, overexpression of PNRC activates, whereas down-regulation of PNRC by RNA interference decreases, pol III transcription in MCF7 cells, suggesting that one of the protein's function is to stimulate pol III transcription (Zhou et al., 2007).

Pol III transcription is repressed by p53. On type 1 and 2 promoters, p53 inhibits pol III promoter occupancy by targeting the TBP subunit of Brf1-TFIIIB (Cairns and White, 1998, Crighton et al., 2003, Stein et al., 2002). For example, after treatment of cells with methane methylsulfonate (MMS), a DNA damaging agent, tRNA transcription is inhibited, at least in part as a result of p53-mediated effects on TFIIIB; the TFIIIB association with TFIIIC2 and pol III in solution is reduced, as is the TFIIIB association with tRNA promoters in vivo (Crighton et al., 2003). On type 3 promoters, p53 inhibits pol III transcription by association with the promoter itself, probably through protein-protein interactions with members of the transcription initiation complex (Gridasova and Henry, 2005).

A second repressor of pol III transcription is the protein Maf1, which was originally identified in S. *cerevisiae* by the isolation of a temperature-sensitive mutation, *maf1-1* (Murawski et al., 1994). In *maf1-1* cells, tRNA levels were elevated, and pol III transcription was much more active in extracts from such cells than in extracts from wild-type cells, suggesting that Maf1 represses pol III transcription (Pluta et al., 2001). Indeed, yeast Maf1 turns out to be an essential common component of at least three signaling pathways that lead to pol III transcription repression, the secretory defect signaling pathway, the target of rapamycin (TOR) signaling pathway, and the DNA damage signaling pathway (Upadhya et al., 2002), see (Willis et al., 2004) for a review).

In cells lacking Maf1, repression of pol III transcription in response to these signaling pathways fails.

Recent work (Oficjalska-Pham et al., 2006, Roberts et al., 2006) has significantly advanced our understanding of repression by Maf1 in yeast (see [Geiduschek and Kassavetis, 2006] for a review). In actively growing yeast cells, Maf1 is present in both the nucleus and the cytoplasm, and a large fraction of Maf1 is phosphorylated, at least in part by PKA, whose activity counteracts Maf1 repression (Moir et al., 2006). Upon exposure of the cells to various stresses, Maf1 is dephosphorylated in a manner dependent on PP2A and translocates to the nucleus, where it occupies pol III promoters as determined by genome-wide chromatin immunoprecipitations (Oficjalska-Pham et al., 2006, Roberts et al., 2006). The dephosphorylated form of Maf1 can associate with pol III and *in vitro*, Maf1 is capable of preventing assembly of a transcription complex by binding to Brf1. This suggests that in addition to inhibiting transcription through binding to pol III, Maf1 can also prevent the assembly of new transcription complexes (Desai et al., 2005).

Maf1 is conserved in other species (Pluta et al., 2001), raising the important possibility that Maf1 might be involved in pol III repression in mammalian cells. Indeed, recent results show that human Maf1 is a repressor of pol III transcription both *in vitro* and *in vivo* (Reina et al., 2006), down-regulating transcription from type 1, 2, and 3 pol III promoters. Human Maf1 is necessary for efficient down-regulation of pol III transcription after stress, and associates with Brf1 as well as with the pol III subunits RPC1 and RPAC2. Like the yeast protein, human Maf1 is phosphorylated and becomes largely dephosphorylated after stress, and it is the dephosphorylated form of Maf1 that associates with pol III (Reina et al., 2006). Thus, Maf1 is potentially a central regulator of pol III transcription in mammalian cells.

From the brief summary above, it is clear that a large number of factors can individually regulate pol III transcription. However, we are still missing a picture of how these various factors interplay to modulate pol III transcription under various conditions and at different times during

the cell cycle. Indeed, we lack information on how the actions of these factors integrate into complex regulatory pathways, and such pathways might simply be too elaborate to be fully described by traditional reductionist approaches. However, with genomic era techniques, it is possible, for example, to correlate the binding of any factor to all pol III (and other) promoters in the genome with transcription efficiency, and this under a variety of conditions. Combinations of such genome-scale analyses with global proteomic analyses will lead to a comprehensive description of these complex regulatory networks, and in turn to a better understanding of cell proliferation and growth constraints.

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