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A mutation in the largest (catalytic) subunit of RNA polymerase II and its relation to the arrest of the cell cycle in G_1 phase

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Abstract

Transcriptional activity of RNA polymerase II is modulated during the cell cycle. We previously identified a temperature-sensitive mutation in the largest (catalytic) subunit of RNA polymerase II (RPB1) that causes cell cycle arrest and genome instability. We now characterize a different cell line that has a temperature-sensitive defect in cell cycle progression, and find that it also has a mutation in RPB1. The temperature-sensitive mutant, tsAF8, of the Syrian hamster cell line, BHK21, arrests at the non-permissive temperature in the mid-G_1 phase. We show that RPB1 in tsAF8 – which is found exclusively in the nucleus at the permissive temperature – is also found in the cytoplasm at the non-permissive temperature. Comparison of the DNA sequences of the *RPB1* gene in the wild-type and mutant shows the mutant phenotype results from a (hemizygous) C-to-A variation at nucleotide 944 in one *RPB1* allele; this gives rise to an ala-to-asp substitution at residue 315 in the protein. Aligning the amino acid sequences from various species reveals that ala^{315} is highly conserved in eukaryotes. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: RPB1; Temperature-sensitive mutant; Hemizygosity; Sequencing

1. Introduction

RNA polymerase II (pol II) is a multi-subunit enzyme responsible for the transcription of most genes in higher eukaryotes (Roeder, 1996; Lee and Young, 2000). It forms enormous complexes, or holoenzymes, that associate with other proteins involved in related functions like splicing, polyadenylation, and the repair of damage in DNA (Hampsey and Reinberg, 1999; Holstege and Young, 1999). In turn, several such holoenzymes are organized into even larger structures called transcription 'factories' (Cook, 1999). At the heart of each individual polymerizing complex lies the catalytic subunit, RPB1; this is the largest subunit found in the core enzyme, and it has homology with the β' subunit of the bacterial enzyme.

Transcriptional activity of pol II is modulated during the cell cycle; the enzyme becomes inactive during mitosis, and – perhaps surprisingly – cells expressing specific mutations in the enzyme or its associated transcription factors arrest at particular points in the cell cycle (reviewed by Bregman et al., 2000). For example, one subunit of the general transcription factor TFIID – $TAF_{II}250$ – was originally identified as a cell cycle control gene, *CCG1*; the temperature-sensitive mutation in *CCG1* found in the Syrian hamster cell line, ts13, can prevent exit from the G_1 phase. Moreover, a different temperature-sensitive mutant of the CHO-K1 line known as tsTM4 contains a mutation in *RPB1* that also interferes with progression through the cycle (Tsuji et al., 1990; Mita et al., 1995; Sugaya et al., 1997, 1998); these cells arrest in the S phase at the non-permissive temperature, and have increased levels of sister chromatid exchanges (Tsuji et al., 1990). Genomic fragments containing the entire coding region of the human *RPB1* gene – as well as the corresponding cDNA tagged with green fluorescent protein – complement the temperature-sensitive defect in tsTM4 (Mita et al., 1995; Sugaya et al., 2000). This defect results from a point mutation (a C-to-T transition) that changes the amino acid residue at position 1006 from a pro to a ser (Sugaya et al., 1997). Proline^{1006} proves to be highly conserved throughout eukaryotes, and substituting the homologous residue in *Schizosaccharomyces pombe*

Abbreviations: A, adenosine; Br-U, bromouridine; BSA, bovine serum albumin; C, cytidine; CDS, coding sequence; CTD, carboxyl-terminal domain; nt, nucleotide; PBS, phosphate-buffered saline; pol II, RNA polymerase II; RPB1, the largest subunit of RNA polymerase II; SDS, sodium dodecyl sulfate; T, thymidine; UTR, untranslated region

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with a serine also led to genome instability and defects in cell cycle progression (Sugaya et al., 1998).

Here, we characterize the defect in another temperature-sensitive mammalian cell, tsAF8. This is a temperature-sensitive mutant of the BHK21 cell line that arrests at the non-permissive temperature in the mid-G_1 phase (Meiss and Basilico, 1972; Burstin et al., 1974); it is also deficient in pol II activity (Rossini and Baserga, 1978; Rossini et al., 1980). Normal growth at the non-permissive temperature can be restored by introducing wild-type chromosomes by cell fusion, or by direct microinjection of purified pol II (Ingles and Shales, 1982; Weachter et al., 1984). Note that a plasmid encoding human RPB1 tagged with green fluorescent protein (Sugaya et al., 2000) is able to complement the deficiency in tsAF8 at 39°C (data not shown). We find that the mutation results from a C-to-A transition that gives an ala-to-asp substitution at position 315 in RPB1. This provides a second example of a mutation in pol II that directly affects progression around the cell cycle.

2. Materials and methods

2.1. Cells

The temperature-sensitive mutant, tsAF8, of the Syrian hamster line, BHK21, was kindly provided by Dr R. Baserga. Cells were grown in Dulbecco's modification of Eagle's medium plus 10% fetal calf serum, 2 mM L-glutamine and antibiotics (GibcoBRL).

2.2. Indirect immunolabeling and microscopy

Transcriptional activity was monitored by indirect immunolabeling and microscopy as described previously (Jackson et al., 1998; Pombo et al., 1999). In brief, cells were grown on glass cover slips for 24 h at 34 or 39°C, then incubated with 1 mM bromouridine (Br-U) for 20 min, washed with PBS and fixed (20 min, 4°C) with 4% paraformaldehyde in 250 mM HEPES (pH 7.4). Fixed cells were permeabilized (20 min, 4°C) with 0.2% Triton X-100 in PBS, blocked using PBS containing 40 mM glycine, 1% BSA, and 0.2% gelatin, before indirect immunolabeling with Cy3 or FITC. The following primary antibodies were used: mouse anti-BrdU (clone MD5110; Caltag), mouse monoclonal directed against the C-terminal domain (CTD) of RPB1 (clone 7C2; Besse et al., 1995), and a mouse monoclonal directed against the phosphorylated form of the CTD (clone H5; BabCo). Images were collected using a YOKOGAWA CSU10 'confocal' microscope and 'contrast-stretched' using Adobe Photoshop.

2.3. Sequencing

The sequences of *RPB1* in wild-type and mutant were obtained using a strategy described previously (Sugaya et al., 1997). Messenger RNAs were prepared from the two cell lines and converted to cDNAs; then, different segments of the cDNAs were amplified using a set of nine primer pairs (primers 52, A, B, C, D, E, F, G, H, I, J, K, L, M, N, 33, 32, oligo $(dT)_{20}$; Sugaya et al., 1997) plus eight additional primers (nucleotide positions of 5′ and 3′ ends are shown in parentheses): P, 5′(259)-CACATCGAACTGGCCAAACCTGTG-(282)3′; Q, 5′(1065)-CATTAGATTCCCTCGAACCCGTCC-(1042)3′; R, 5′(3615)-CCGAATATCTCCTTGGCTGCTGCG-(3638)3′; S, 5′(4417)-GGTCAAAACAGCCAGTGCCCGCTG-(4394)3′; T, 5′-(5191)-TCTCCAAGCTACTCGCCAACTTCTCCAAGT-(5220)3′; U, 5′(5819)-TAAGTGGAGCCCTTGGGGGAGGTG-(5796)3′; W, 5′(5599)-TATTCACCTACATCTCCCAAGTATTCTCCT-(5628)3′; 34C, 5′(5882)-TCAGCCCTGATGACAGTGATGAGG-(5905)3′. Next, PCR products were treated with exonuclease I and shrimp alkaline phosphatase (PCR products pre-sequencing reagent pack; Amersham Pharmacia Biotech), directly sequenced (ABI Prism BigDye Terminator Cycle Sequencing Kit; Applied Biosystems) using an 'ABI Prism 3700' sequencer, and sequences were assembled using AutoAssembler (Applied Biosystems). More than three independent samples of mRNAs were prepared from both wild-type and mutant cells. Genomic DNA from the two sources was also amplified and directly sequenced using the same approach.

3. Results and discussion

3.1. The distribution of RPB1 in tsAF8 changes at the non-permissive temperature

As the nature of the temperature-sensitive mutation in tsAF8 cells was unclear, we first checked their transcriptional activity at 34 and 39°C. Living cells were grown in Br-U, and the amount and distribution of the resulting Br-RNA was monitored by microscopy after indirect immunolabeling. Many small nucleoplasmic foci and fewer larger nucleolar foci containing Br-RNA were seen in both wild-type (BHK21/13) and mutant (tsAF8) cells, and these patterns did not change after growth for 24 h at the non-permissive temperature (Fig. 1A,B,G,H). This is consistent with previous results showing that mutant cells grow normally for 1 day at 39°C, before they begin to die (Rossini et al., 1980; data not shown).

We next monitored the distribution of RPB1 by indirect immunolabeling. RPB1 contains an essential carboxy-terminal domain (CTD) composed of 52 tandem repeats of a heptapeptide with the consensus sequence tyr-ser-pro-thr-ser-pro-ser; ser^2 and ser^5 in this heptapeptide are often phosphorylated *in vivo* (Dahmus, 1996; Komarnitsky et al., 2000). Antibodies are available that recognize the hyperphosphorylated CTD, or both the phosphorylated and unphosphorylated CTD. Thus, monoclonal antibody H5 recognizes the phospho-ser^2 version of RPB1 (Bregman et

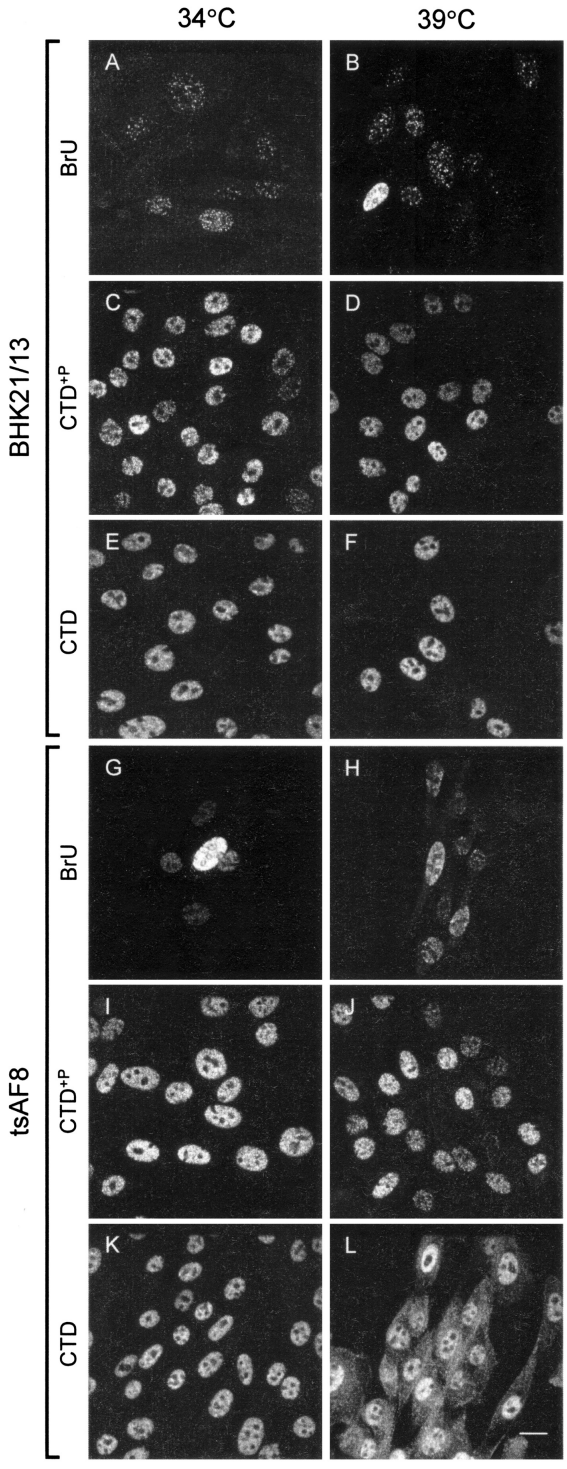

Fig. 1. Altered distribution of RPB1 in the temperature-sensitive mutant at 39°C. Wild-type BHK21/13 or mutant tsAF8 cells were grown at 34 or 39°C for 24 h, regrown ±1 mM Br-U for 20 min, and fixed; next, Br-RNA, and the phosphorylated and total fractions of the CTD of RPB1, were indirectly immunolabeled (with Cy3, FITC, and Cy3, respectively), and single equatorial sections were collected using a 'confocal' microscope. Bar, 20 μm. (A,B,G,H) Nascent Br-RNA is concentrated in small nucleoplasmic foci, and a few larger nucleolar foci. (C,D,I,J) Phosphorylated RPB1 is found in many small foci in the nucleoplasm, but not in nucleoli or cytoplasm. (E,F,K,L) RPB1 distributions in wild-type and mutant are similar at 34°C, but growth at 39°C induces cytoplasmic labeling (L).

al., 1995; Kim et al., 1997). It yields many small bright foci in the nucleoplasm, but not in nucleoli or the cytoplasm (Fig. 1C,D,I,J). Although patterns were similar at 34 and 39°C in wild-type and mutant, the intensity of labeling in the mutant was slightly reduced at 39°C (compare Fig. 1I with Fig. 1J). The 7C2 antibody recognizes both the phosphorylated and unphosphorylated forms of the CTD (Besse et al., 1995). It gave similar patterns in the wild-type at 34 and 39°C (Fig. 1E,F), and in the mutant at 34°C (Fig. 1K). However, growing the mutant at 39°C led to the spread of some labeling from the nucleoplasm to the cytoplasm (Fig. 1L), perhaps because the mutant subunit cannot be incorporated at the non-permissive temperature into larger structures like the core or holoenzyme, or a transcription factory. It remains possible that the phenotype could result from the altered distribution.

3.2. The sequence of RPB1 in wild-type and mutant cells

The sequence of cDNAs encoding RPB1 in wild-type (i.e. BHK21/13) and mutant cells (i.e. tsAF8) was determined using a strategy and primers used previously with a different set of cells (i.e. wild-type CHO-K1 and mutant tsTM4; Sugaya et al., 1997). Although the nucleotide sequence for rpb1 of Syrian hamster was slightly different from that of Chinese hamster (see Fig. 2A; 95.9% identities in CDS, 93.6% in 5′ untranslated region (UTR), 90.1% in 3′ UTR), we have cloned and sequenced almost the full length of rpb1 cDNA successfully. The 6612 nt sequence of the wild-type *RPB1* in BHK21/13 that was obtained has been deposited as Accession number AB052229 in the DDBJ, GenBank, and EMBL databases. Since we had determined two other nucleotide sequences of mammalian rpb1, the obtained nucleotide sequence and its deduced amino acid sequence were compared with those of Chinese hamster (CHO-K1; Sugaya et al., 1997), human (Mita et al., 1995) and mouse (Ahearn et al., 1987). The similarities in the coding sequences (CDS) – as well as those of the 5′ and 3′ UTRs – are illustrated in Fig. 2 (amino acid sequence was essentially the same; data not shown). As expected, CDSs are more conserved than the UTRs, and analysis of the phylogenetic trees suggests that the speed of nucleotide substitution in the 3′ UTR was roughly three times faster than in the CDS.

Comparison of wild-type and mutant (tsAF8) sequences revealed one consistent difference. Direct sequencing of cDNAs prepared from the pool of mRNAs in the wild-type always gave a C at nucleotide 944; however, sequencing the equivalent cDNAs from the mutant gave both a C and an A (Fig. 3A). This suggests that the mutant contains two alleles, with one being similar to the wild-type and the other with a C-to-A transition at nucleotide 944 (Fig. 3A). This difference was confirmed in two ways. First, similar results were obtained with two different preparations of mRNAs from both wild-type and mutant cells. Second, the relevant genomic regions in the wild-type and mutant

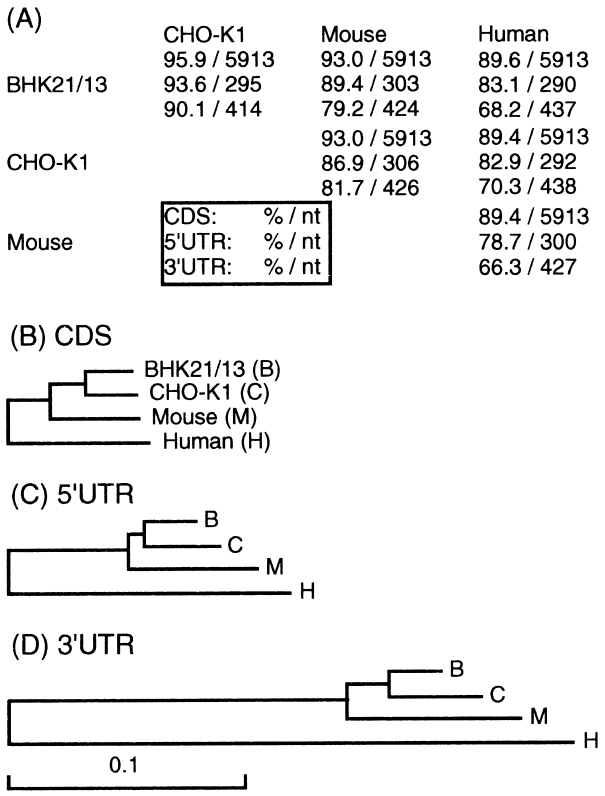

Fig. 2. Comparison of the sequences of mammalian *RPB1* genes. (A) The percentage of identical nucleotides and the number of nucleotides (%/nt) of the coding sequence (CDS), and the 5′ and 3′ UTRs. (B–D) Phylogenetic trees (prepared using the N-J method; Saitou and Nei, 1987) of the CDS, 5′ UTR, and 3′ UTR of BHK21/13 (GenBank/EMBL/DDBJ Accession number: AB052229), CHO-K1 (D87294; D87293 for 3′ UTR), mouse (M14101; M12130), and human (X63564); branch length indicates evolutionary distance.

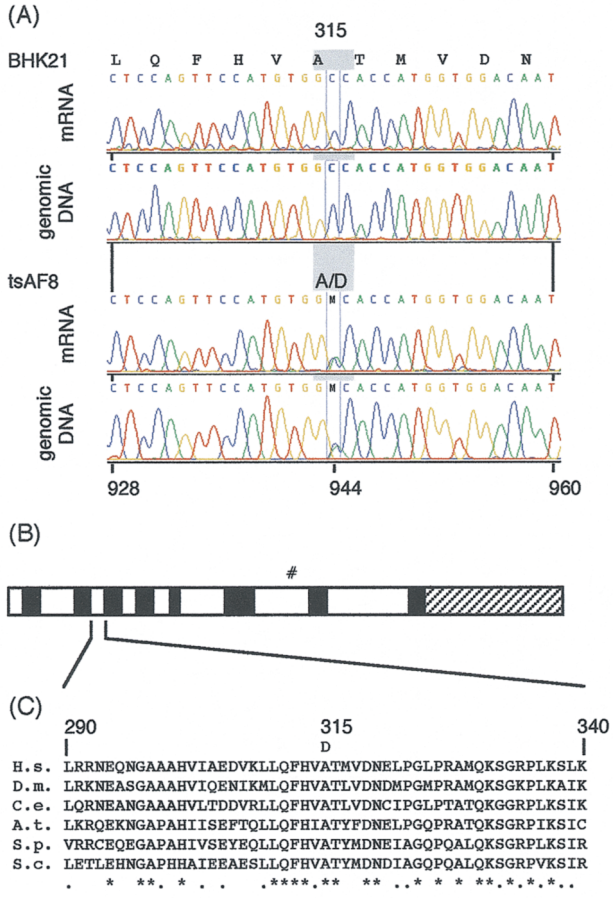

Fig. 3. Sequences around the mutation in tsAF8. (A) Sequencing traces obtained with *RPB1* DNA (prepared by reverse transcription of pools of mRNA, or from genomic DNA) from wild-type BHK21 and mutant tsAF8 cells. The wild-type gives a C at nucleotide 944, while the mutant gives a mixture of C and A. A C-to-A transition at nucleotide 944 converts ala^{315} to asp. (B) The structure of mammalian *RPB1*. Black boxes, regions conserved with the β′ subunit of the bacterial enzyme; hatched box, heptapeptide repeats in the CTD; #, the mutation in tsTM4 (Sugaya et al., 1997). (C) Protein sequences of the region containing the mutation in tsAF8. Alignments were made using CLUSTAL W (Thompson et al., 1994) relative to positions 290–340 in human RPB1 (SWISS-PROT Accession number: P24928; sequences of mouse, Chinese hamster, and Syrian hamster are identical) using sequences from *Drosophila melanogaster* (P04052), *Caenorhabditis elegans* (P16356), *Arabidopsis thaliana* (P18616), *Schizosaccharomyces pombe* (P36594), and *Saccharomyces cerevisiae* (P04050). Asterisks, identical residues; dots, residues belonging to the same physicochemical group.

were amplified using primers ‘B’ and ‘Q’ (see Section 2.3), and sequenced; the resulting traces showed that position 944 contained a C in one case, and both a C and an A in the other (Fig. 3A). Hemizygosity is consistent with the slow appearance of the mutant phenotype at the non-permissive temperature, and with findings using somatic cell hybrids (Shales et al., 1980).

The C-to-A transition results in an ala-to-asp substitution at position 315 in the protein. Ala^{315} is highly conserved throughout eukaryotes (Fig. 3C), even though it lies outside the regions that are similar to those found in the β′ subunit of the bacterial enzyme (shown as black boxes in Fig. 3B). It remains to be seen where ala^{315} lies within the three-dimensional structure of the core enzyme (Cramer et al., 2000).

4. Conclusion

We conclude that the temperature-sensitive phenotype seen in tsAF8 cells is determined by a hemizygous variation at nucleotide 944 (C to A) in one of the two *RPB1* alleles; the C-to-A transition results in (i) an ala-to-asp substitution at position 315 in the largest subunit of pol II, and (ii) an altered distribution of the enzyme at the non-permissive temperature which leads to an accumulation in the cytoplasm. We speculate that mutant RPB1 might fail to be incorporated as efficiently as the wild-type RPB1 into transcription complexes (e.g. holoenzymes, transcription factories), to accumulate in the cytoplasm. As some wild-type RPB1 remains in the nucleus, transcriptional activity is maintained, at least for 1 day. (tsTM4 cells also survive for 24 h at 39°C (data not shown).) Why, then, do several cell cycle mutants turn out to have mutations in pol II (i.e.

tsAF8, tsTM4) or its transcription factors (i.e. ts13)? There are two obvious possibilities: RPB1 may be one of the most important targets for checkpoint control of the cycle, and its full activity may be required for the expression of certain critical regulators of the cell cycle like cyclin D1 (Suzuki-Yagawa et al., 1997).

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References

Ahearn, J.M., Bartolomei Jr., M.S., West, M.L., Cisek, L.J., Corden, J.L., 1987. Cloning and sequence analysis of the mouse genomic locus encoding the largest subunit of RNA polymerase II. J. Biol. Chem. 262, 10695–10705.

Besse, S., Vigneron, M., Pichard, E., Puvion-Dutilleul, F., 1995. Synthesis and maturation of viral transcripts in herpes simplex virus type 1 infected HeLa cells: the role of interchromatin granules. Gene Expr. 4, 143–161.

Bregman, D.B., Du, L., van der Zee, S., Warren, S.L., 1995. Transcription-dependent redistribution of the large subunit of RNA polymerase II to discrete nuclear domains. J. Cell Biol. 129, 287–298.

Bregman, D.B., Pestell, R.G., Kidd, V.J., 2000. Cell cycle regulation and RNA polymerase II. Front. Biosci. 5, 244–257.

Burstin, S.J., Meiss, H.K., Basilico, C., 1974. A temperature-sensitive cell cycle mutant of the BHK cell line. J. Cell. Physiol. 84, 397–408.

Cook, P.R., 1999. The organization of replication and transcription. Science 284, 1790–1795.

Cramer, P., Bushnell, D.A., Fu, J., Gnatt, A.L., Maier-Davis, B., Thompson, N.E., Burgess, R.R., Edwards, A.M., David, P.R., Kornberg, R.D., 2000. Architecture of RNA polymerase II and implication for the transcription mechanism. Science 288, 640–649.

Dahmus, M.E., 1996. Reversible phosphorylation of the C-terminal domain of RNA polymerase II. J. Biol. Chem. 271, 19009–19012.

Hampsey, M., Reinberg, D., 1999. RNA polymerase II as a control panel for multiple coactivator complexes. Curr. Opin. Genet. Dev. 9, 132–139.

Holstege, F.C.P., Young, R.A., 1999. Transcriptional regulation: contending with complexity. Proc. Natl. Acad. Sci. USA 96, 67–72.

Ingles, C.J., Shales, M., 1982. DNA-mediated transfer of an RNA polymerase II gene: reversion of the temperature-sensitive hamster cell cycle mutant TsAF8 by mammalian DNA. Mol. Cell. Biol. 2, 666–673.

Jackson, D.A., Iborra, F.J., Manders, E.M., Cook, P.R., 1998. Numbers and organization of RNA polymerases, nascent transcripts, and transcription units in HeLa nuclei. Mol. Biol. Cell 9, 1523–1536.

Kim, E., Du, L., Bregman, D.B., Warren, S.L., 1997. Splicing factors associate with hyperphosphorylated RNA polymerase II in the absence of pre-mRNA. J. Cell Biol. 136, 19–28.

Komarnitsky, P., Cho, E.J., Buratowski, S., 2000. Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription. Genes Dev. 14, 2452–2460.

Lee, T.I., Young, R.A., 2000. Transcription of eukaryotic protein-coding genes. Annu. Rev. Genet. 34, 77–137.

Meiss, H.K., Basilico, C., 1972. Temperature sensitive mutants of BHK 21 cells. Nat. New Biol. 239, 66–68.

Mita, K., Tsuji, H., Morimyo, M., Takahashi, E.-I., Nenoi, M., Ichimura, S., Yamauchi, M., Hongo, E., Hayashi, A., 1995. The human gene encoding the largest subunit of RNA polymerase II. Gene 159, 285–286.

Pombo, A., Jackson, D.A., Hollinshead, M., Wang, Z., Roeder, R.G., Cook, P.R., 1999. Regional specialization in human nuclei: visualization of discrete sites of transcription by RNA polymerase III. EMBO J. 18, 2241–2253.

Roeder, R.G., 1996. Nuclear RNA polymerases: role of general initiation factors and cofactors in eukaryotic transcription. Methods Enzymol. 272, 165–171.

Rossini, M., Baserga, R., 1978. RNA synthesis in a cell cycle-specific temperature sensitive mutant from a hamster cell line. Biochemistry 17, 858–863.

Rossini, M., Baserga, S., Huang, C.H., Ingles, C.J., Baserga, R., 1980. Changes in RNA polymerase II in a cell cycle-specific temperature-sensitive mutant of hamster cells. J. Cell. Physiol. 103, 97–103.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425.

Shales, M., Bergsagel, J., Ingles, C.J., 1980. Defective RNA polymerase II in the G1 specific temperature sensitive hamster cell mutant TsAF8. J. Cell. Physiol. 105, 527–532.

Sugaya, K., Sasanuma, S., Nohata, J., Kimura, T., Hongo, E., Higashi, T., Morimyo, M., Tsuji, H., Mita, K., 1997. Cloning and sequencing for the largest subunit of Chinese hamster RNA polymerase II gene: identification of a mutation related to abnormal induction of sister chromatid exchanges. Gene 194, 267–272.

Sugaya, K., Ajimura, M., Tsuji, H., Morimyo, M., Mita, T., 1998. Alternation of the largest subunit of RNA polymerase II and its effect on chromosome stability in Schizosaccharomyces pombe. Mol. Gen. Genet. 258, 279–287.

Sugaya, K., Vigneron, M., Cook, P.R., 2000. Mammalian cell lines expressing functional RNA polymerase II tagged with the green fluorescent protein. J. Cell Sci. 113, 2679–2683.

Suzuki-Yagawa, Y., Guermah, M., Roeder, R.G., 1997. The ts13 mutation in the TAF(II) 250 subunit (CCG1) of TFIID directly affects transcription of D-type cyclin genes in cells arrested in G1 at the nonpermissive temperature. Mol. Cell. Biol. 17, 3284–3294.

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.

Tsuji, H., Matsudo, Y., Tsuji, S., Hanaoka, F., Hyodo, M., Hori, T., 1990. Isolation of temperature-sensitive CHO-K1 cell mutants exhibiting chromosomal instability and reduced DNA synthesis at nonpermissive temperature. Somat. Cell Mol. Genet. 16, 461–476.

Weachter, D.E., Avignolo, C., Freund, E., Riggenbach, C.M., Mercer, W.E., McGuire, P.M., Baserga, R., 1984. Microinjection of RNA polymerase II corrects the temperature-sensitive defect of tsAF8 cells. Mol. Cell. Biochem. 60, 77–82.