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C-Myc Deregulation During Transformation Induction: Involvement of 7SK RNA

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Abstract The process of oncogenic transformation has been widely studied but is still poorly understood. We have focused on the mechanism of deregulation of the c-myc gene during transformation of a temperature-sensitive SV40-transformed mouse cell line. Run-on transcription assays showed that the two c-myc minor promoters, P1 and P3, are transiently activated following induction of transformation and that peak activation of both promoters is preceded by a large increase in transcription of a small RNA (7SK). To test the possibility that this RNA might participate in promoter activation, we transfected cells with sense and antisense oligodeoxynucleotides corresponding to different regions of the 7SK RNA predicted to be accessible within the RNP particle. Out of 14 oligos tested, inhibition of activation of P1 and/or P3 was observed with four antisense oligonucleotides corresponding to looped regions in the putative 7SK secondary structure. To identify c-myc promoter sequences which might serve as targets for 7SK activity, we carried out mobility-shift assays with either whole or 7SK-depleted cell extracts. The CT element of the c-myc promoter formed a 7SK-dependent complex which could be competed only with the same antisense 7SK oligo that suppressed P1 and P3 activation in vivo. Taken together these results suggest that 7SK RNP participates in transformation-dependent c-myc deregulation. J. Cell. Biochem. 64:313–327.

Key words: c-myc promoter utilization; SV40-induced transformation; transcription; temperature-sensitive cells; 7SK RNA

The concept of chromatin-remodelling factors and transcription factors that are RNA-based has been recently revived with the discovery of RNAs that appear to act at the DNA level. Examples are the transcription factor TF III R, which controls tRNA transcription in the silk worm [Dunstan et al., 1994a,b], the RNA product of the XIST gene which controls X-chromosome inactivation [Brockdorff et al., 1992; Penny et al., 1996], and the H19 RNA which can act as a tumor suppressor [Hao et al., 1993]. Previous results from our own and other laboratories have suggested an RNA involvement in the transcriptional control of two oncogenes, the SV40 T-antigen and the c-myc genes, both of which encode protein transcription factors important in oncogenic transformation. In our laboratory, indirect evidence pointed to one particular type of small RNA, 7SK, as a possible participant in transcription regulation. 7SK is an abundant, highly conserved class III RNA originating from one or more genes belonging to a family of interspersed repeats in the genome (Alu II in human and B2 in mouse) [Zieve et al., 1977; Gurney and Eliceiri, 1980; Reichel and Benecke, 1980; Murphy et al., 1984, 1987; Gallant et al., 1987; Moon and Krause, 1991]. Although a number of studies on 7SK RNA and associated proteins have been carried out in other laboratories [for review see Reddy and Busch, 1988], the function of this abundant small RNA remains unknown. Early in vitro assays revealed that gel-purified 7SK RNA from SV40-transformed cells activated overall transcription initiation in normal nuclei [Ringuette et al., 1980, 1982]. Northern blot hybridizations between total small RNAs and various oncogene probes showed that the 7SK band hybridized selectively with promoter regions of both the SV40 and the mammalian but not chicken c-myc genes. This promoter and species selectivity likely reflects an interaction with these promoters and their trans-acting factors [Sohn et

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al., 1983; Kurz et al., 1988]. Furthermore, the intensity of the hybridization signals varied in direct proportion to transformation and tumorigenicity of the cell lines tested [Krause et al., 1986]. These results led to the hypothesis that 7SK RNA may participate in the transformation process by regulating transcriptional initiation of specific genes such as c-myc.

The c-myc protooncogene is known to play an important role in the determination of the various physiological fates of a cell, including proliferation, arrest, differentiation, and death. Deregulation of the gene, therefore, results in pathological states that appear to be a general feature of many types of cancers [reviewed in Marcu et al., 1992]. Thus, we have focused our attention on deregulation of c-myc gene expression following induction of transformation in temperature-sensitive mouse and human cell lines. Use of the temperature-sensitive cell lines avoids problems often encountered when comparing normal and transformed cell lines or tumors since observed changes in permanently transformed cells could be due, in part, to gene amplification, chromosomal rearrangements, or other secondary phenomena. Moreover, the more rapid proliferation exhibited by transformed cells makes it difficult to dissociate transformation-specific from cell cycle–specific events. In contrast, the temperature-sensitive cell lines, upon induction, slow down rather than accelerate their cell cycle progression [Gallant et al., 1989; Luo and Krause, 1994]. Thus, any observed changes are likely to be related to transformation rather than proliferation.

We started by comparing c-myc and c-ras mRNA levels at various times following transfer of SV40 Ts(A)-transformed mouse cells to the permissive temperature. Northern blot hybridizations revealed substantial increases in both transcripts, starting at 30 min postinduction and maintained thereafter [Gallant et al., 1989]. However, nuclear run-on experiments revealed a peak of c-myc transcription at 2 h postinduction, preceded by a sevenfold activation of class III RNAs at 30 min postinduction and followed by c-ras activation 6 h after induction [Gallant et al., 1989]. To determine whether the temporal appearance of these different classes of RNAs was part of a gene cascade, we initiated a more detailed analysis of transcription from each of the known c-myc promoters.

The mammalian c-myc gene locus is known to contain four promoters. In normal cells the major promoter, P2, accounts for 75–90% of c-myc mRNA, P1 accounts for 10–25%, while P0 and P3 together account only for 5% of c-myc transcripts [Eick et al., 1990]. To quantify any changes in transcription from each of these promoters, occurring during transformation, we developed a quantitative assay utilizing reverse transcription followed by polymerase chain reaction (RT-PCR). Application of this method to mouse and human cells, transformed by a temperature-sensitive mutant of the SV40 virus, enabled us to detect a three- to fivefold increase in P1- and P3-initiated transcripts within 30 min of transformation induction in both cell lines [Luo and Krause, 1994]. This higher level of transcription was maintained for 24 h postinduction and was also observed in stably transformed cells. The major P2-initiated transcripts were found not to be significantly affected. However, the level of exon III–containing c-myc mRNAs increased gradually up to 24 h postinduction and was also found to be high in stably transformed cells [Luo and Krause, 1994]. The mechanism responsible for the gradual increase in c-myc mRNA was therefore unclear, given that the two minor promoters still contributed relatively little to the total c-myc mRNA population. These results indicated an unpredicted complexity in the deregulatory process of oncogenic transformation, which is likely to involve alterations at various levels of promoter accessibility, transcription initiation, attenuation, and posttranscriptional processing.

In the present investigation we extend these studies to further explore the mechanism of c-myc deregulation during transformation induction. This study had two main objectives: 1) to discriminate between promoter activity and transcript stabilization as determinants of the observed increased levels of c-myc transcripts and 2) to search for factors which might be involved in the process. Through a combination of nuclear run-on transcription, oligo transfection, and electrophoretic mobility-shift assays (EMSA) with whole or affinity-depleted extracts, we show that transformation induction involves transient activation of both P1 and P3 c-myc promoters. We also present evidence that a 7SK RNP is involved in the activation of at least one of these promoters at early stages of oncogenic induction.

METHODS

Cell Strains and Culture Conditions

The SV40 Ts(A)-transformed mouse 3T3 cells, line A 255 [Alwine et al., 1977], the wild-type SV40-transformed Balb 3T3 cells (SVT2), and Balb 3T3 cells (American Type Culture Collection, Rockville, MD) were cultivated in Dulbecco's modified Eagle's medium (GIBCO-BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (ICN Biomedicals, Costa Mesa, CA). The temperature-sensitive cells were maintained at 33°C until approximately 2 weeks before experiments. The cells were then transferred to 39°C and subcultured for a few passages before transfer to 33°C and harvested at the times indicated.

Run-On Transcription Assays

All 50 mer antisense oligonucleotide probes used to detect relative activity from each promoter and all other oligonucleotides used for transfection assays were synthesized in a Cyclone Plus DNA synthesizer (Milligen/Biosearch, Burlington, MA). The 50 mer sequences were as follows: P1 (+106 to +57) 5′ CTTCTTTTTCCCGCCAAGCGTCGGCGAACGCTGCCTTCAGGAGGCAGGA 3′;P2(+381to+332)5′CTGCAATGGGCAAAGTTTCCCAGCGGCGGCGAGGGTTGCGGCCGCTGATG 3′; UP3 (+944 to +895) 5′ ACGCAAAAGGTAATCCCTTCTCCAAAGACCTCAGGATTCCAAGGGCTTTC 3′; P3 (+1,906 to +1,857) 5′ CTACTATCAGTGACGCTCGTCGGACTAGCAGCTGCTCCGAGTCCCCGCCT 3′; E2 (+2,507 to +2,458) 5′ GTAGATGATGTTCTTGATGAAGGTCTCGTCGTCAGGATCGCAGATGAAGC 3′; 7SK (+185 to +136) 5′ ACCGAAGACCGGTCCTCCTCTATTCGGGGAAGGTCGTCCTCTTCGACCGA 3′; GAPDH (+500 to +451) 5′ CCAAAGTTGTCATGGATGACCTTGGCCAGGGGGGCTAAGCAGTTGGTGGT 3′. The c-myc mouse sequences are positioned relative to the P1 start site [Stanton et al., 1984] as illustrated under the c-myc map in Figure 1. The glyceraldehyde-3 phosphate dehydrogenase (GAPDH) probe was selected as per the sequence reported by Sabath et al. [1990] and the 7SK probe from the sequence reported by Moon and Krause [1991]. M13 phage DNA was used as negative control. All probes were denatured in 50% formamide, 7.4% formaldehyde, 1× SSC at 65°C for 15 min, neutralized by addition of 2 volumes of 20× SSC, and applied to Duralose-UV™ membranes (Stratagene, La Jolla, CA) at a concentration of 1 µg/slot (Hybri-Slot™ manifold; Gibco/BRL). Nuclei were isolated essentially as described [Greenberg and Ziff, 1984]. Aliquots of 6×10^7 cells were washed three times with saline and spun down at 500*g* for 5 min. Pellets were resuspended in 1 ml ice-cold lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% (v/v) NP40) and incubated on ice for 5 min. Nuclei were spun down at 500*g* for 5 min at 4°C, washed in the same buffer, resuspended in 300 µl ice-cold storage buffer (50 mM Tris-HCl, pH 8.3, 40% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA), and frozen in liquid nitrogen until use. Run-on transcription assays in the presence of [^{32}P] UTP (ICN) were as described [Xu et al., 1991]. RNAs were extracted by the hot phenol chloroform method [Soeiro and Darnell, 1969], precipitated three times, and hybridized in 50% formamide, 5× SSCPE, 1× Denhardt's solution, 0.1% SDS, 500 µg/ml yeast tRNA, and 20 µg/ml salmon sperm DNA, at 42°C for 72 h. Washes were in 2× SSC, 1% SDS at 42°C, two times, followed by 2× SSC, 10 µg/ml RNase A at 37°C for 30 min, and again twice in 2× SSC, 1% SDS at 37°C. Filters were dried and exposed to Kodak X-OMAT AR film for various time periods.

Quantitation of the signals was calculated from scans of selected film exposures using a GS300 Transmittance/Reflectance Scanning Densitometer (Hoefer Instruments, San Francisco, CA). The linear response range of the film was first determined to ensure that quantitation was limited to this range in each of the exposures selected. Densitometric data were captured and analysed using Maxima 820 software (Waters, Millipore Products Division, Milford, MA). The signals were standardized for ^{32}P decay, exposure time, and reference to the signal obtained from GAPDH by use of the following formula: standardized signal = peak density of scan/[^{32}P decay × exposure time × GAPDH (33°/39°)].

Oligonucleotide Transfections and Assays

Sense and antisense oligonucleotides for the various regions of 7SK RNA were based on the sequences reported for mouse 7SK [Moon and Krause, 1991]. The sense sequences, with the numbers indicating position relative to the transcription start site (+1), are as follows: +7–27: 5′ GAGGGCGATCTGGCTGCGACA 3′; +29–49: 5′ CTGTCACCCCATTGATCGCCA 3′; +77–

94: 5′ AGGCGGGTGTCCCCTTCC 3′; +95–114: 5′ TCCCTCACCGCTCCATGTGC 3′; +177–194: 5′ GTCTTCGGTCAAGGGTAT 3′; +221–241: 5′ ACCTCCAAACAAGTCCTCAA 3′; +284–300: 5′ CCAGACACATCCAAATG 3′. For comparison of the effects of all these sense and corresponding antisense 7SK oligonucleotides, we used a previously reported RT-PCR assay utilizing promoter-specific c-myc primers [Luo and Krause, 1994]. For this assay, A255 cells were cultured in microtiter plates at 39°C and exposed to 40 μM of each oligo for 8 h before transfer to 33°C and assayed 2 h later. Poly(A)$^+$ RNAs were isolated using Hybond™ mAP paper (Amersham, Arlington Heights, IL) as described [Luo and Krause, 1994] except that about 5,000 cells were lysed on each 2 mm^2 of paper. Radioactive RT-PCR with [^{32}P]dCTP was carried out in a single step using Retrotherm reverse transcriptase (Epicentre Technologies, Madison, WI) and primers specific for detection of P1, P3, and GAPDH as described [Luo and Krause, 1994]. The amplified fragments were loaded in duplicate, separated in 2% agarose gels containing 0.4 μg/ml ethidium bromide, and photographed under UV. Bands corresponding to P1, P3, and GAPDH amplification products were excised from the gel and dried at 70°C overnight, and radioactivities were determined. Oligo effects were calculated from the counts per minute ratios of c-myc to GAPDH, obtained from the excised bands, and percent inhibition of transcription by each of the oligos tested was calculated using the following formula: % inhibition = (C-T)33° × 100/(C33°–C39°) where T stands for counts per minute ratio of P1/GAPDH or P3/GAPDH in transfected cells, while C stands for the same ratio in control cells at either 33 or 39°C.

Transfection with K7-27 antisense oligo was also assayed by nuclear run-on transcription. In this case transfection conditions were identical except that aliquots of 10^7 cells were used for nuclear isolation and run-on assays as above.

Electrophoretic Mobility Shift Assays (EMSA)

To test the possible participation of 7SK RNA in complex formation at the promoter level, we used a 25 mer c-myc probe representing the purine-rich strand in the CT box region of the mouse c-myc P1 promoter (−151 to −175 relative to the P1 start site [Bernard et al., 1983]: 5′ GGGGAGCCGGGGAAAGAGGAGGAGG 3′. The oligo was labelled with ^{32}P using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Hela cell extracts were prepared as described [Ausubel et al., 1989], and 10^5 cpm of the probe was incubated with 20–40 mg of extract in a final volume of 25 μl in binding buffer (10 mM Hepes, pH 7.9, 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 1 mM DTT, 2 mg dI-dC), for 30 min at room temperature. Complexes were resolved on 5% polyacrylamide gels as described (Ausubel et al., 1989). Depletion of 7SK RNP from the extracts was carried out overnight at 4°C in streptavidin-agarose (Upstate Biotechnology, Lake Placid, NY) to which biotinylated antisense K 95-114 oligo was bound. Mock-depleted extracts were treated with avidin-agarose in the same manner in the absence of bound oligo.

RESULTS

Transformation Induction Involves Transient Activation of P1 and P3 Promoters

To test whether the accumulation of P1- and P3-initiated transcripts observed upon induction of transformation in temperature-sensitive cells [Luo and Krause, 1994] is due to promoter activation, we carried out nuclear run-on transcription assays using 50 mer antisense oligodeoxynucleotides as probes. The position of those probes within the c-myc map, their target transcripts, and an example of the results of the run-on assays are depicted in Figure 1. It is apparent that both P1 and P3 promoters are activated only transiently, being detected as early as 30 min postinduction and reverting to normal levels by 24 h. P0 is not detectable by this assay and is therefore not included. Although the signals for P2 are likely to be affected by the increased transcription from P1, those for P3 do not appear to be derived from upstream-initiated transcripts since the upstream probe (UP3) detects only traces of transcripts in all conditions tested. The appearance of sizable exon II signals at 39°C and after 24 h at 33°C is puzzling given that no intron I signals (UP3) or P3 initiated transcripts are detectable at these times. However, these results are typical for c-myc run-on assays (see also 3T3 vs. SVT2), and other authors have interpreted this to mean that the processivity of the polymerase is lowest in intron I and highest in exon I [Yankulov et al., 1994]. However, other interpretations are possible, and one should not exclude the possibility that rapid splicing and intron degradation during the run-on assay may be taking place. Evidence for cotranscriptional splicing has been recently presented [Wuarin and

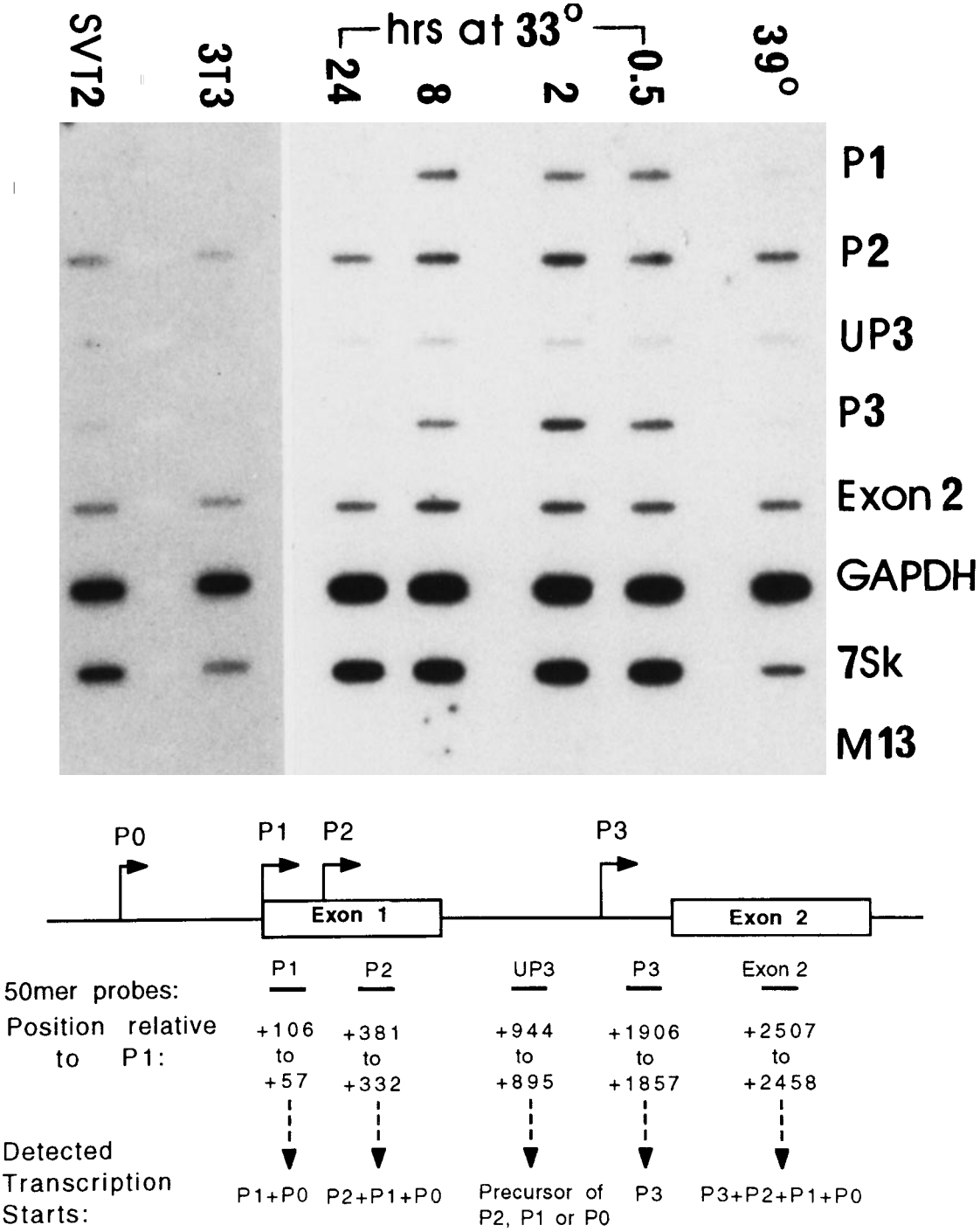

Fig. 1. Run-on transcription assays for comparison of relative activity of 7SK and c-myc promoters as a function of time after the temperature switch (39° to 33°C) in temperature-sensitive A255 mouse cells. Stably transformed cells (SVT2) are also compared with their nontransformed counterparts (3T3). The position of antisense 50 mer oligonucleotide probes for detection of c-myc run-on transcripts is depicted under the c-myc map, together with the respective transcripts detected. Arrows indicate the start sites of the four c-myc promoters, P0 to P3.

Schibler, 1994]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signals (overexposed in this film) are constant, but 7SK RNA transcription activation appears to be immediate and is sustained beyond 24 h since it is detectable in SV40-transformed mouse 3T3 cells (SVT2) as compared with their nontransformed counterparts (3T3).

To quantitate the relative output from P1, P3, and 7SK promoters as a function of time following the temperature switch, we analyzed

various exposures of the X-ray films obtained in run-on assays by scanning densitometry as described in Methods. We also included extra time points (5 and 12 h) for more precise determination of peak c-myc transient activation. The results are presented in Figure 2. It is apparent that a sevenfold peak activation of 7SK at 30 min appears concomitantly with activation of P1 and P3 transcription, which reaches a peak at around 5 h postinduction. Both c-myc promoters are seen to revert to normal activity by 24 h, and no changes are evident between normal and stably transformed cells. Thus, the sustained higher levels of c-myc mRNA previously found to follow transformation [Luo and Krause, 1994] suggest postinitiation effects, such as increased read through from the P2 promoter, differential processing, and/or differential stabilization of c-myc transcripts.

Antisense 7SK Oligos Inhibit P1 and P3 Promoter Activation

The sequential activation of 7SK and c-myc minor promoters during transformation, together with previous hints for its possible role as a trans-acting factor, prompted us to design an experimental test for its participation in c-myc promoter activation in vivo. Computer (FASTA) searches identified regions of limited homology between 7SK and c-myc P1 and P3 promoter elements. On the basis of these homologies and the reported accessibility of some 7SK RNA sequences within the 7SK ribonucleoprotein (RNP) particle [Wassarman and Steitz, 1991], we synthesized oligodeoxynucleotide sequences (see Methods; Table I) corresponding to presumed exposed regions of the RNA, with the expectation that some of the antisense probes might base-pair with 7SK RNA and therefore interfer with its activity during transformation induction. Sense and antisense oligonucleotides for seven of these regions were transfected into A255 cells prior to the induction switch, and P1- and P3-initiated transcripts were analyzed by RT-PCR 2 h postinduction using the same primers previously described [Luo and Krause, 1994]. Inclusion of

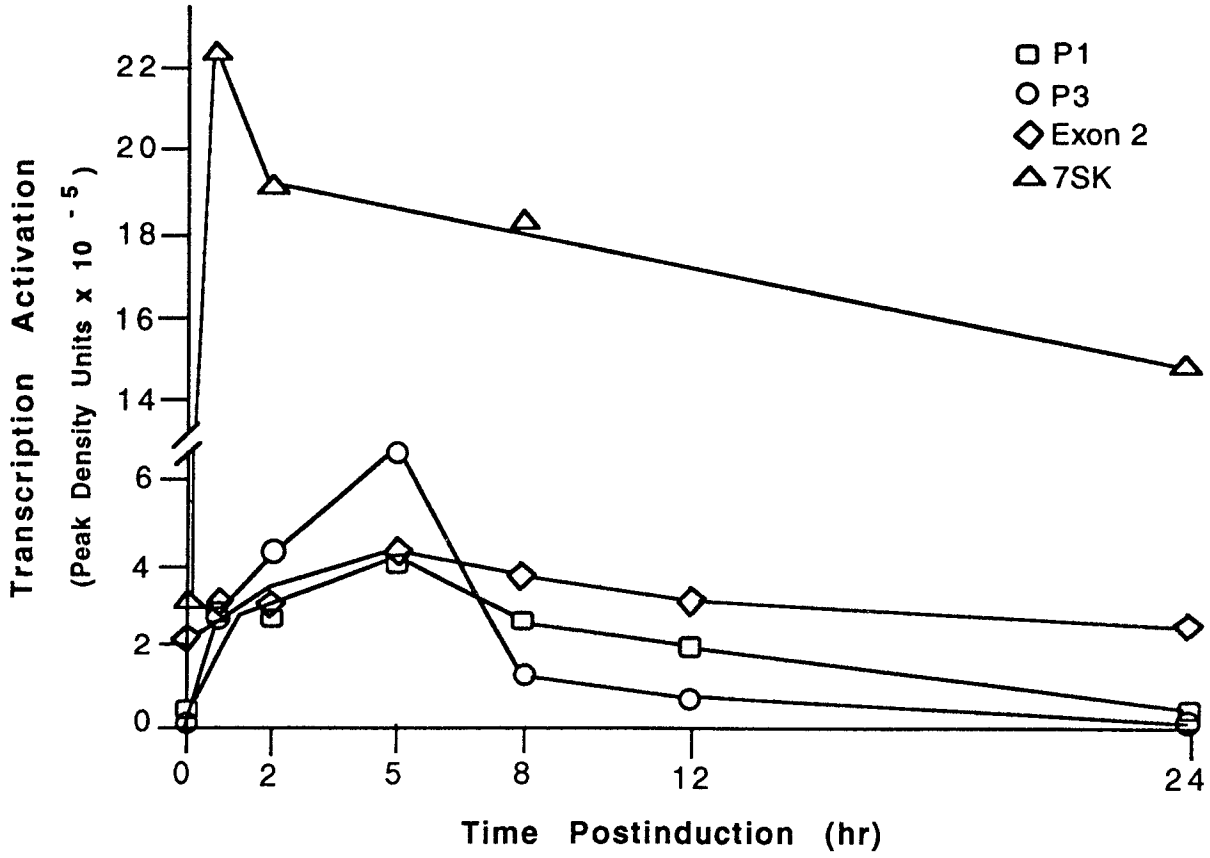

Fig. 2. Scanning densitometric analysis of P1, P3, exon 2, and 7SK signals obtained in run-on experiments, as depicted in Fig. 1, for quantitation of promoter activation as a function of time after transformation induction in temperature-sensitive A255 cells. Each curve was obtained from scans of selected exposures of the X-ray film, as detailed in Methods.

TABLE I. Comparison of Activity, Accessibility, and Secondary Structure of Selected RNA Sequences Within 7SK RNP

			Activity[b]	
Oligo position	% cleavage[a]	Loop region	P1	P3
7-27	≥50	Yes	0	+++
29-49	≥50	No	0	0
77-94	ND	No	0	0
95-114	ND	Yes	+++	++
177-194	≥50	Yes	+	0
221-241	100	Yes	0	+
284-300	≥50	No	0	0

[a]Percent cleavage was determined in nuclear extracts by sensitivity to RNase H in the presence of complementary oligodeoxynucleotides as reported [Wassarman and Steitz, 1991]. ND, not determined.

[b]Relative activity is estimated from the percent inhibition of P1 or P3 transformation-induced activation by antisense 7SK oligonucleotides as illustrated in Figure 4.

GAPDH primers in each reaction served as internal control.

The effects of three of the oligo pairs—K7-27, K77-94, and K95-114—on P1 and P3 switch-dependent activation are illustrated in Figure 3. It is apparent that oligo K7-27 depresses P3 activation, oligo K95-114 depresses both P1 and P3, and oligo K77-94 has no effect on either. The GAPDH levels remain unchanged in all transfected cultures. For both the K7-27 and the K95-114 oligos, only the antisense versions had an effect. The effect of all seven pairs of 7SK oligonucleotides on P1- and P3-initiated transcription was calculated by quantitation of the respective radioactive PCR signals relative to GAPDH controls as described in Methods and is presented in Figure 4. It is apparent that only four of the antisense 7SK oligonucleotides have a significant inhibitory effect on the level of switch-dependent P1- and/or P3-initiated transcripts. In addition to K95-114, which affects both P1 and P3 transcripts, K177-194 affects only P1, while K7-27 and K221-241 affect only P3. Variations below ±20% could not be detected visually and were judged insignificant. These results suggest that more than one region of 7SK is involved in transcription control. Different regions of 7SK RNA may act in concert to provide transcript-specific effects. Alternatively, some antisense oligos may cause misfolding of the RNA, which in turn may affect RNP assembly, structure, and function.

In Figure 5 we outline the position of both active (open symbols) and inactive (closed symbols) 7SK regions on the putative secondary structure of the RNA, and Table I compares the accessibility, loop structure, and activity of the 7SK RNA sequences used for the transfection experiments. Although the proposed folding may not represent the RNA as it exists in combination with proteins in the RNP particle, it is interesting to note that all active regions contain loops, while the inactive ones lie within base-paired stems.

Since detection of 7SK RNA effects on c-myc transcription by RT-PCR does not allow discrimination between regulatory activity at either the transcriptional or posttranscriptional levels, we carried out further experiments using a run-on transcription assay to measure transcription rates in the presence and absence of an active antisense oligo. For these experiments we selected the one oligo that showed the highest inhibition in the RT-PCR experiments. Antisense oligo K7-27, which eliminated the rise in P3-initiated transcripts, was used to transfect cells to detect its effect in run-on transcription 5 h postinduction. The results are illustrated in Figure 6. K7-27 severely inhibited P3 promoter activation, which remained near the level seen in noninduced cells. The level of GAPDH remained constant.

Possible C-Myc P1 Promoter Target for 7SK RNP

We next looked for specific elements in the c-myc promoters that might be involved in 7SK-dependent activation following transformation induction. Computer searches for regions with limited homology to the active regions of 7SK revealed several possible targets for the K7-27 sequence on the P3 promoter and a single match with the K95-114 in the CT box region of the P1 promoter (12 out of 17 bases). This region, −151 to −175 bp relative to P1, lies within a sequence showing a strong homopurine/homopyrimidine strand asymmetry. This is one of the three sequences whose hypersensitivity to DNase correlates with active transcription of the human c-myc gene [Michelotti et al., 1995]. The CT box region in the human c-myc gene consists of five imperfect direct repeats of the sequence CCCTCCCCA. This CT element has been shown to confer a five- to tenfold stimulation of transcription to heterologous promoters, both in vitro and in vivo [Takimoto et al., 1993; DesJardins and Hay, 1993]. The same region has also been shown to assume an H-conformation in vitro, consisting of a triplex/single-stranded DNA structure [Kinniburgh, 1989].

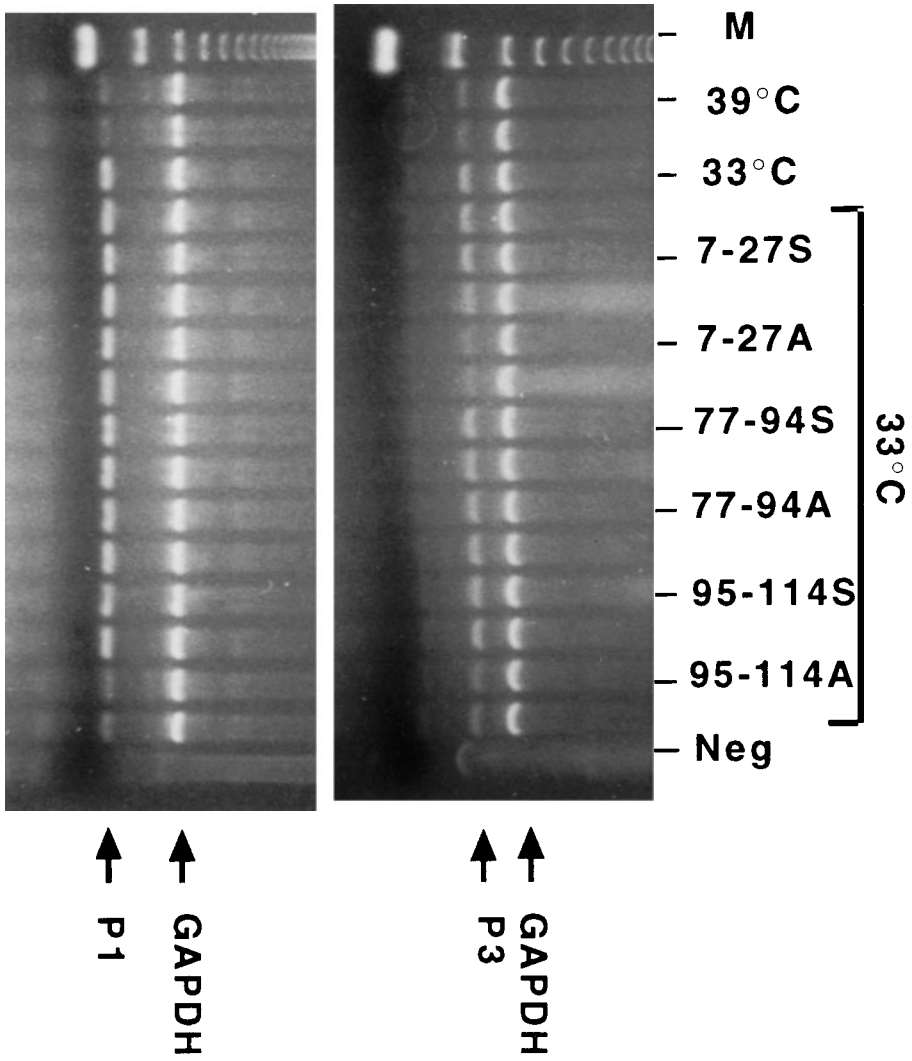

Fig. 3. Effect of transfected sense and antisense 7SK oligonucleotides on the activation of c-myc P1 and P3 promoters in A255 cells as detected by RT-PCR 2 h after switching to the permissive temperature of 33°C. The uninduced state (39°C) and untransfected induced cells (33°C) are shown as controls. Neg, no template negative control; M, size markers (BRL 123 ladder). Numbered intervals for each oligo indicate position within the 7SK sequence (numbered 5′, 1–331); S, sense oglios; A, antisense oligos.

Importantly, this structure has been found to bind to an unknown RNP factor in electrophoretic mobility-shift assays [Davis et al., 1989]. Thus, we decided to test the equivalent, well-conserved region of the mouse c-myc gene [Bernard et al., 1983] to investigate the possibility that the unknown RNP factor seen to bind this promoter element might contain 7SK RNA.

Figure 7 (lanes 2–4) illustrates the EMSA results obtained with Hela nuclear extracts probed with the 25 nucleotide oligo corresponding to the mouse CT element as detailed in Methods. Biotinylated anti-7SK oligo 95-114 was coupled to avidin-agarose beads and used to remove 7SK RNPs. This treatment completely inhibited complex formation. Extracts treated in the same manner with uncoupled avidin-agarose (lane 5) formed complexes identical to those formed with untreated extracts. Three complexes appear in the control lanes; however, only the lower one (indicated by thick arrow) appeared to be specific and was competed exclusively by KA 95-114, the same antisense K oligo which inhibited both P1 and P3 activation in vivo. Neither the inactive 7SK antisense oligo, KA 77-94 (lanes 6–9), nor the sense KS 95-114 (lanes 14–17), interfered with this complex. Other random oligos also failed to

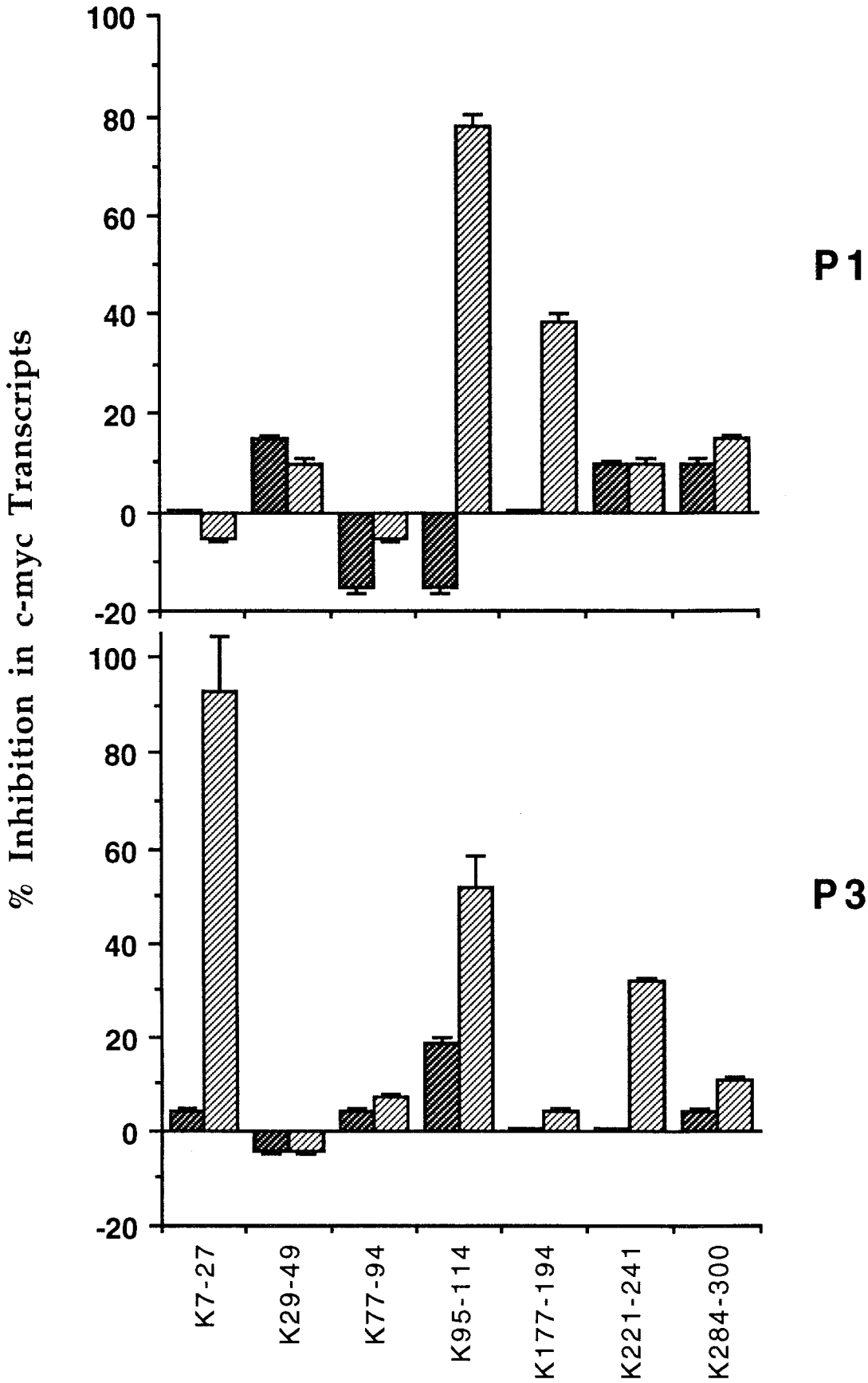

Fig. 4. Quantitation of the effect of transfected 7SK oligonucleotides on switch-induced activation of P1 and P3 promoters in A255 cells 2 h after transformation induction (39° to 33°C). The count per minute ratios of c-myc to GAPDH, obtained from bands excised from agarose gels such as that depicted in Fig. 3, are expressed as percent inhibition in transfected cells as compared to nontransfected controls (see Methods). Lighter hatched bars, antisense oligos; darker stippled bars: sense oligos. Standard deviations calculated from four repeated experiments are indicated.

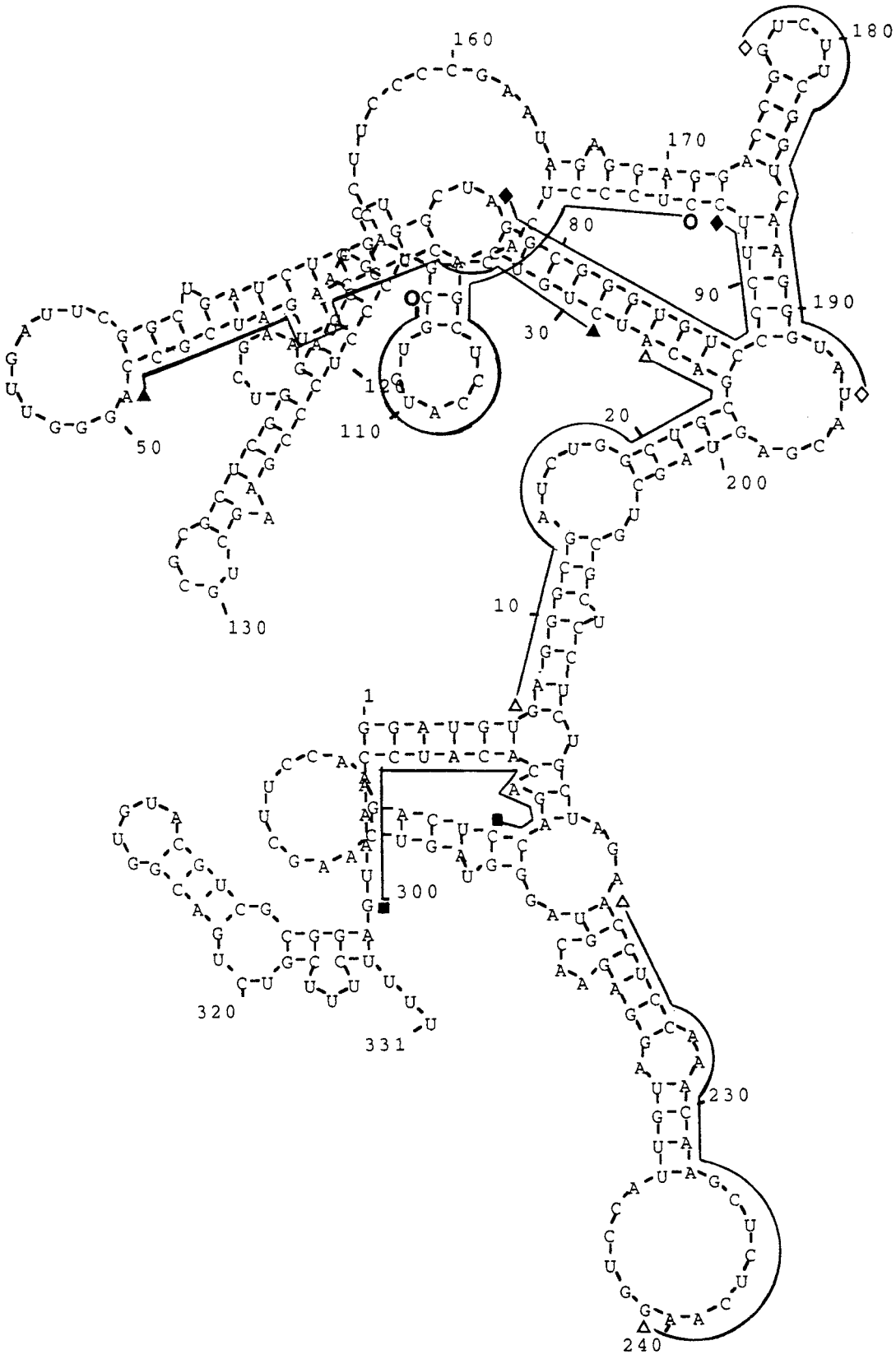

Fig. 5. Putative structure of 7SK RNA showing the position of the seven oligo pairs (sense and antisense) used for transfection. The structure was obtained using the DNASIS Pro software (Hitachi, San Bruno, CA). Open symbols flank the outlined active sequences, and closed symbols flank the inactive ones.

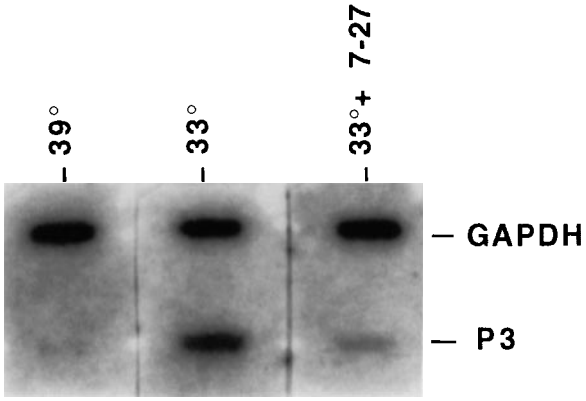

Fig. 6. Effect of antisense 7SK oligo (7-27) on P-3 promoter activity detected in a run-on transcription assay 5 h after switching A255 cells to the permissible temperature of 33°C. 39°, noninduced cells; 33°, induced cells; 33° + 7-27, induced cells in the presence of the 7SK oligo. The positions of P3 and GAPDH probes are indicated.

interfere with formation of the specific complex (not shown).

We next examined the sensitivity of this complex to both proteases and RNases. The results are illustrated in Figure 8. Proteinase K (lanes 3–5) removes all complexes, while RNase A (lanes 9, 10) interferes only with the lower complex, confirming it as the RNA-dependent one. As a further control, we added an RNase inhibitor (RNasin; Promega, Madison, WI) to RNase A–treated extracts and found that this restored the specific complex (lane 11). RNase H, however, had no effect on complex formation (lanes 6–8), indicating that Watson and Crick base pairs between 7SK and the c-myc probe are either not involved or are protected within the complex.

DISCUSSION

Transcription Activation by 7SK RNP

We have utilized both run-on transcription and RT-PCR assays to detect transformation-induced changes in c-myc gene expression that occur in temperature-sensitive mouse cells immediately following transfer to the permissive temperature. The finding of a transient activation of two minor c-myc promoters, and the fact that activation of these promoters is preceded by a substantial rise in the transcription of 7SK RNA, provided the ideal system to test the hypothesis that this RNA is involved in c-myc deregulation during transformation induction.

We had previously developed a quantitative RT-PCR assay and applied it to the measurement of the relative output of c-myc transcripts produced from each of its four promoters as a function of the physiological state of the cells. The method not only permits the use of small numbers of cells but was shown to be superior to the more classical S1-nuclease or RNase protection assays in that it provides more reliable quantitation of minor promoters [Luo and Krause, 1994]. This method allowed us to quantitate the inhibitory effect of transfected antisense 7SK oligonucleotides on the transformation-induced elevated levels of c-myc P1- and P3-initiated transcripts. We used seven 7SK oligonucleotide pairs selected on the basis of their accessibility within the 7SK RNP particle [Wassarman and Steitz, 1991]. We also considered in particular those regions of the 7SK sequence which showed some homology with P1 and P3 promoter elements and might therefore interact with those elements. Oligo-transfected cells were subjected to RT-PCR to identify four antisense oligonucleotides which inhibited the increase in P1 and/or P3 transcript accumulation during transformation induction. Significantly, all of the active oligos were complementary to exposed looped regions of the RNA; those located on base-paired stems showed no effect. Neither did any of the seven 7SK sense oligonucleotides tested. To discriminate between promoter-specific effects and those acting postranscriptionally, we extended the RT-PCR approach to show that the same results could be obtained by run-on transcription assays using the most active of the 7SK antisense oligonucleotides. These results support the hypothesis that 7SK RNA is involved in the activation of transcription in vivo. Our next question concerned the identification of promoter sites which may act as targets for 7SK activity.

Likely Promoter Target for 7SK RNP

To test whether 7SK RNP does form specific complexes with c-myc promoter sequences, we resorted to electrophoretic mobility-shift assays. Early studies with c-myc identified six candidate regulatory regions hypersensitive to DNase I digestion [Siebenlist et al., 1984; Dyson et al., 1985]. In three of these regions, hypersensitivity correlated with transcription activation in several cell lines. One of these sites, termed III_1, is situated upstream of the P1 promoter and contains the CT box element which has been found to stimulate transcrip-

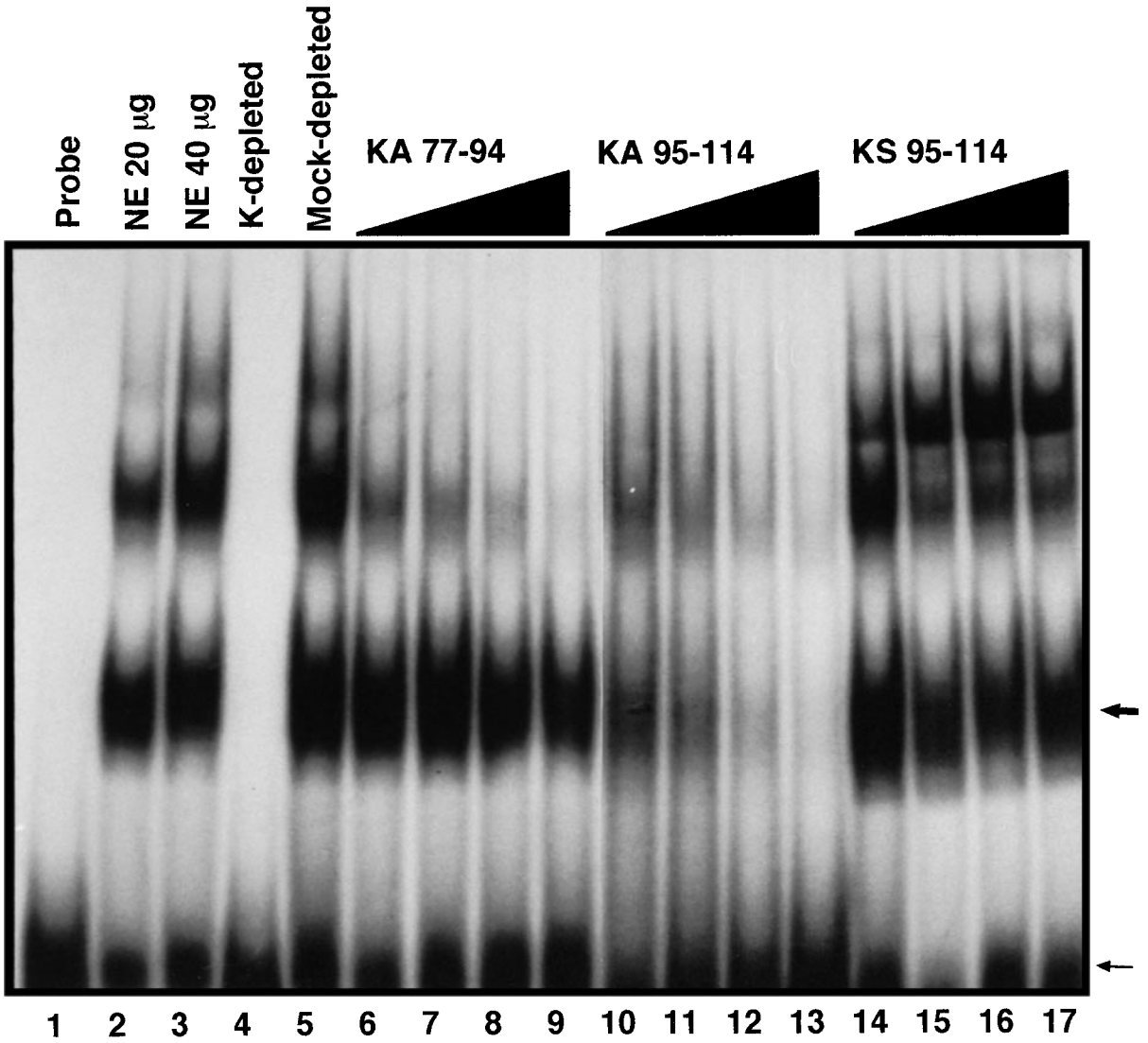

Fig. 7. Competition EMSA using a 25 mer corresponding to the c-myc purine-rich strand of the CT element of the P1 promoter as probe with 7SK-depleted and undepleted Hela cell extracts. NE, normal whole cell extract; K-depleted, extract treated with avidin-agarose–coupled antisense 7SK oligo 95-114; Mock-depleted, treated with uncoupled avidin-agarose in the same manner. Competition was carried out in undepleted extracts (40 mg) with increasing amounts of competitor antisense (KA) or sense (KS) oligos, with numbers representing their respective position in the 7SK sequence. The proportions of competitor to probe were 25×, 50×, 100×, and 200×, respectively. The thick arrow indicates the position of the specific complex, while the thin one indicates that of the free probe.

tion of heterologous promoters both in vivo and in vitro [Takimoto et al., 1993; DesJardins and Hay, 1993]. We selected this site as a possible target for 7SK RNP for several reasons: 1) the CT box contains the best contiguous homology match (12 out of 17 bases) with the same region of 7SK RNA which appeared to be involved in both P1 and P3 activation during transformation induction; 2) triplex-forming oligonucleotides have been shown to bind to this region and have been suggested to alter gene expression [Cooney et al., 1988; Durland et al., 1991; Merghy et al., 1991; Postel et al., 1991]; 3) the strong homopurine/homopyrimidine strand asymmetry of this region of the human promoter has been found to lead to an H-DNA conformation in vitro and to bind to an endogenous RNP in EMSA with Hela cell extracts. The endogenous RNP interaction was postulated to stabilize the H structure in vivo [Kiniburgh, 1989; Davis et al., 1989].

Our EMSA results, using the equivalent purine-rich strand of the mouse c-myc promoter in whole or 7SK-depleted Hela extracts,

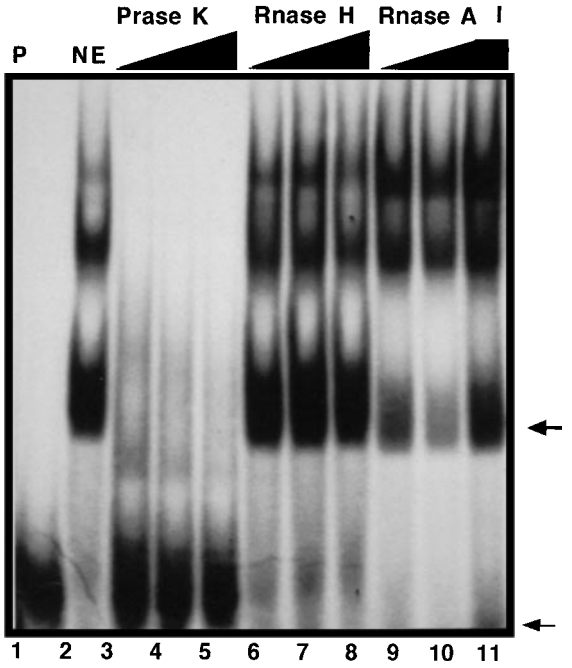

Fig. 8. Sensitivity of the EMSA complex to proteinase K and RNases. P, probe; NE, 40 mg of whole extract; Prase K, 5, 10, and 20 mg of proteinase K, respectively, were incubated with the extract and probe as described in Methods; Rnase H, 4, 8 and 16 units RNase H were added in the same manner; RNase A, 4 and 8 mg RNase A were added; I, 8 mg of RNase A was added in the presence of 400 units of RNasin. Thick and thin arrows indicate the position of the complex and free probe, respectively.

identify the endogenous unknown RNP of Davis et al. [1989] as 7SK RNP. A 7SK-specific complex was found to correspond to that of Davis et al. [1989] and was similarly sensitive to RNase A. However, in contrast with the earlier work, the complex was found to be insensitive to RNase H, indicating that Watson/Crick base pairs are either not involved or are protected within the complex. We consider, in addition, two other possibilities: 1) the RNP may be involved in a purine/purine/pyrimidine triple helix with the CT element, or 2) the RNP may not bind directly to the promoter and may instead compete for binding of other factors which normally contribute to the relative inaccessibility of this element in the c-myc promoter. The binding of 7SK RNP might induce a conformational change, thus permitting the binding of transcription factors.

7SK and the Mechanism of Transformation Induction

The use of the temperature-sensitive cell line permits us to follow the sequence of events that takes place during transformation induction. Previous experiments have proved that the temperature shift per se has no effect when applied to normal or stably transformed cells [Gallant et al., 1989; Luo and Krause, 1994]. Since the temperature-sensitive defect in the SV40 A gene does not prevent the accumulation of its protein product [Brockman, 1978], it is likely that the shift to the permissive temperature results in functional activation of preexisting T antigen molecules which are known to activate transcription from class III genes such as 7SK [Singh et al., 1985]. This RNA in turn may participate in the selective activation of c-myc P1 and P3 promoters. Whether 7SK RNP binds directly to these promoters remains to be investigated. Although the results of EMSA are suggestive of a direct interaction with the purine-rich strand of the c-myc CT element, it is also possible that the RNP particles act in a more indirect fashion. For example, they might participate in a more general chromatin remodelling process during transformation induction which exposes the minor promoters for immediate interaction with trans-acting factors. It is not known how transient activation of these minor promoters can contribute to the increased levels of c-myc mRNA since, even at their maximum activity, they still contribute relatively little to the total mRNA population [Luo and Krause, 1994]. Several possibilities can be considered: 1) P1 and P3 activation might lead to an increased accessibility of the c-myc P2 promoter for the binding of transcription activators; 2) promoter elements of related genes may likewise be affected, contributing to the overall effect; 3) such processes may increase the read-through transcription from the major promoter; 4) processing changes may take place, such as a different 3′ end formation which can result in the production of more stable messengers [Bonieu et al., 1988; Bernstein et al., 1992].

Transient activation has been reported for hormone-inducible promoters where hormone-dependent loading of transcription factors at the promoter is lost after 24 h of continuous hormone treatment [Lee and Archer, 1994]. The authors suggest that transient promoter activation correlates with nucleosomal remodelling in chromatin. Evidence to date indicates that transcriptional activation requires that transcription factors successfully compete with histones for binding to promoters [Felsenfeld, 1992]. It is possible that 7SK RNP may act as a facilitator

in this process. The abundance of 7SK RNP in the nucleus and the finding that the level of transcription of its RNA remains elevated in transformed cells predicts that this RNP is broadly utilized in regulating other nuclear events. Likely, 7SK RNP is only one of many ubiquitous factors which may be recruited during oncogenesis.

Despite extensive research, the c-myc gene continues to yield a seemingly inexhaustible supply of complexities. The present results offer new clues to its deregulation during transformation induction and present the first concrete suggestion for a functional involvement of an RNA of hitherto unknown function. However, much work remains to be done to unravel the precise mechanisms responsible for transformation-induced accumulation of c-myc mRNA in unrearranged genomes.

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REFERENCES

Alwine JC, Reed ST, Stark GR (1977): Characterization of the autoregulation of simian virus 40 gene A. J Virol 24:22–29.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1989): "Current Protocols in Molecular Biology." New York: John Wiley and Sons.

Bernard O, Cory S, Gerondakis S, Webb E, Adams JM (1983): Sequence of the murine and human cellular myc oncogenes and two modes of myc transcription resulting from chromosomal translocation in B lymphoid tumors. EMBO J 2:2375–2383.

Bernstein PL, Herrick DJ, Prokipcak RD, Ross J (1992): Control of c-myc messenger RNA half life in vitro by a protein capable of binding to a coding region stability determinant. Genes Dev 6:642–654.

Bonieu A, Piechaczyc M, Marty L, Cuny M, Blanchard JM, Fort P, Jeanteur P (1988): Sequence determination of c-myc mRNA turnover: Influence of 3′ and 5′ noncoding regions. Oncogene Res 3:155–166.

Brockdorff N, Ashworth A, Kay GF, McCabe VM, Morris DP, Cooper PJ, Swift S, Rastan S (1992): The product of the mouse Xist gene is a 15 Kb inactive X-specific transcript containing no conserved ORF and located in the nucleus. Cell 71:515–526.

Brockman WW (1978): Transformation of Balb c 3T3 cells by ts(A) mutants of simian virus 40: Temperature sensitivity of the transformed phenotype and retransformation by wild type virus. J Virol 25:860–870.

Cooney M, Czernuszewicz G, Postel EH, Flint SJ, Hogan ME (1988): Site-specific oligonucleotide binding represses transcription of the human c-myc gene in vitro. Science 241:456–459.

Davis TL, Firulli AB, Kinniburgh AJ (1989): Ribonucleotide and protein factors bind to an H-DNA–forming c-myc element: Possible regulators of the c-myc gene. Proc Natl Acad Sci U S A 86:9682–9686.

DesJardins E, Hay N (1993): Repeated CT elements bound by zinc finger proteins control the absolute and relative activities of the two principal human c-myc promoters. Mol Cell Biol 13:5710–5724.

Dunstan HM, Young LS, Sprague KU (1994a): TF III R is an isoleucine tRNA. Mol Cell Biol 14:3588–3595.

Dunstan HM, Young LS, Sprague KU (1994b): $tRNA^{ile}_{IAU}$ (TF III R) plays an indirect role in silkworm class III transcription in vitro and inhibits low frequency DNA cleavage. Mol Cell Biol 14:3596–3603.

Durland RH, Kessler DJ, Gunnell S, Duvic M, Pettitt BM, Hogan ME (1991): Binding of triple helix oligonucleotides to sites in gene promoters. Biochemistry 30:9246–9255.

Dyson PJ, Littlewood TD, Forster A, Rabbitts TH (1985): Chromatin structure of transcriptionally active and inactive human c-myc alleles. EMBO J 4:2885–2891.

Eick D, Polack A, Kofler E, Lenoir GM, Rickinson AB, Bornkamm GW (1990): Expression of P0 and P3 RNA from the normal and translocated c-myc allele in Burkitt's lymphoma cells. Oncogene 5:1397–1402.

Felsenfeld G (1992): Chromatin as an essential part of the transcriptional mechanism. Nature 355:219–224.

Gallant J, Lovely J, Krause MO (1987): Localization of small RNAs hybridizable to a B2 clone in the nuclear fraction of mouse cell lines. Mol Biol Rep 12:49–53.

Gallant J, Kurz J, Krause MO (1989): Increased rates of specific RNA polymerase III transcripts precede overexpression of cellular oncogenes upon induction of transformation. Oncogene Res 4:39–46.

Greenberg ME, Ziff EB (1984): Stimulation of 3T3 cells induces transcription of the c-fos protooncogene. Nature 311:433–437.

Gurney T, Eliceiri GL (1980): Intracellular distribution of low molecular weight RNA species in Hela cells. Proc Natl Acad Sci U S A 87:398–403.

Hao Y, Crenshaw T, Moulton T, Newcomb E, Tycko B (1993): Tumor suppressor activity of H19 RNA. Nature 365:764–767.

Kinniburgh AJ (1989): A cis-acting transcription element of the c-myc gene can assume an H-DNA conformation. Nucleic Acids Res 17:7771–7778.

Krause MO, Kurz J, Lovely J, MacPherson P (1986): Elevated amounts of a 7S nuclear RNA with sequence homology to a tumor virus promoter in transformed and tumorigenic cells. Cancer Res 46:1990–1993.

Kurz J, Lovely J, Cubitt S, Krause MO (1988): Distinct nuclear 7S RNAs hybridize to regulatory regions of two oncogenes. Biochem Biophys Res Commun 152:753–761.

Lee HL, Archer TK (1994): Nucleosome-mediated disruption of transcription factor–chromatin initiation complexes at the mouse mammary tumor virus long terminal repeat in vivo. Mol Cell Biol 14:32–41.

Luo Y, Krause MO (1994): Changes in promoter utilization in human and mouse c-myc genes upon transformation induction in temperature-sensitive cell lines. J Cell Physiol 160:303–315.

Marcu KB, Bossone SA, Patel AJ (1992): Myc function and regulation. Annu Rev Biochem 61:809–860.

Merghy JL, Sun JS, Rougée M, Montenay Garestier T, Barcelo F, Chomilier J, Hélène C (1991): Sequence-specificity in triple-helix formation: Experimental and theoretical studies of the effect of mismatches on triplex stability. Biochemistry 30:9791–9798.

Michelotti EF, Tomonaga T, Krutzsch H, Levens D (1995): Cellular nucleic acid binding protein regulates the CT element of the human c-myc protooncogene. J Biol Chem 270:9494–9499.

Moon IS, Krause MO (1991): Common RNA polymerase I, II and III upstream elements in mouse 7SK gene locus revealed by the polymerase chain reaction. DNA Cell Biol 10:23–32.

Murphy S, Altruda F, Ullu E, Tripodi M, Silengo L, Melli M (1984): DNA sequences complementary to human 7SK RNA show structural similarities to the short mobile elements of the mammalian genome. J Mol Biol 177:575–590.

Murphy S, DiLiegro C, Melli M (1987): The in vitro transcription of the 7SK RNA gene by RNA polymerase III is dependent only on the presence of an upstream promoter. Cell 51:122–126.

Penny GD, Kay GF, Sheardown SA, Rastan S, Brockdorff N (1996): Requirement for Xist in X chromosome inactivation. Nature 379:131–137.

Postel EH, Flint SJ, Kessler DJ, Hogan ME (1991): Evidence that a triplex-forming oligodeoxyribonucleotide binds to the c-myc promoter in Hela cells, thereby reducing c-myc mRNA levels. Proc Natl Acad Sci U S A 88:8227–8231.

Reddy R, Busch H (1988): Structure and function of major and minor small nuclear ribonucleoproteins particles. In Birnstiel ML (ed): Springer-Verlag, pp 1–37.

Reichel R, Benecke BJ (1980): Reinitiation of synthesis of small cytoplasmic RNA species K and L in isolated HeLa cell nuclei. Nucleic Acids Res 8:225–234.

Ringuette MJ, Liu WC, Jay E, Yu KK-Y, Krause MO (1980): Stimulation of transcription of chromatin by specific small nuclear RNAs. Gene 8:211–224.

Ringuette MJ, Gordon K, Szyszko J, Krause MO (1982): Specific small nuclear RNAs from SV40-transformed cells stimulate transcription initiation in nontransformed isolated nuclei. Can J Biochem 60:252–262.

Sabath DE, Broome HE, Phystowsky MB (1990): Glyceraldehyde-3-phosphate dehydrogenase mRNA is a major interleukin 2–induced transcript in a cloned T-helper lymphocyte. Gene 91:185–191.

Siebenlist U, Hennighausen L, Battey J, Leder P (1984): Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell 37:381–391.

Singh K, Carey M, Saragosti S, Botchan M (1985): Expression of enhanced levels of small RNA polymerase III transcripts encoded by the B2 repeats of simian virus 40–transformed mouse cells. Nature 314:553–556.

Soeiro R, Darnell JE (1969): Competitive hybridization by "presaturation" of Hela cell DNA. J Mol Biol 44:551–562.

Sohn U, Szyszko J, Coombs D, Krause MO (1983): 7SK nuclear RNA from simian virus 40–transformed cells has sequence homology to the SV40 promoter. Proc Natl Acad Sci U S A 80:7090–7094.

Stanton LW, Fahrlander PD, Tesser PM, Marcu KB (1984): Nucleotide sequence comparison of normal and translocated murine c-myc gene. Nature 310:423–425.

Takimoto M, Tomonaga T, Matunis M, Avigan M, Krutzsch H, Dreyfuss G, Levens D (1993): Specific binding of heterogeneous ribonucleoprotein-K to the human c-myc promoter in vitro. J Biol Chem 268:18249–18258.

Wassarman DA, Steitz JA (1991): Structural analysis of the 7SK ribonucleoprotein (RNP), the most abundant human small RNP of unknown function. Mol Cell Biol 11:3432–3445.

Wuarin J, Schibler U (1994): Physical isolation of nascent RNA chains transcribed by RNA polymerase II: Evidence for cotranscriptional splicing. Mol Cell Biol 14:7219–7225.

Xu L, Morgenbesser SD, DePinho RA (1991): Complex transcriptional regulation of myc family gene expression in the developing mouse brain and liver. Mol Cell Biol 11:6007–6015.

Yankulov K, Blau J, Purton T, Roberts S, Bentley DL (1994): Transcription elongation by RNA polymerase II is stimulated by transactivators. Cell 77:749–759.

Zieve G, Benecke B, Penman S (1977): Synthesis of two classes of small RNA species in vivo and in vitro. Biochemistry 16:4520–4525.