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An S-phase specific release from a transcriptional block regulates the expression of mouse ribonucleotide reductase R2 subunit

Stefan Björklund, Eva Skogman and Lars Thelander

Department of Medical Biochemistry and Biophysics, University of Umeå, S-901 87 Umeå, Sweden

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Ribonucleotide reductase (RR) activity in mammalian cells is closely linked to DNA synthesis. The RR enzyme is composed of two non-identical subunits, proteins R1 and R2. Both proteins are required for holoenzyme activity, which is regulated by S-phase specific *de novo* synthesis and breakdown of the R2 subunit. In quiescent cells stimulated to proliferate and in elutriated cell populations enriched in the various cell cycle phases the R2 protein levels are correlated to R2 mRNA levels that are low in G_0/G_1-phase cells but increase dramatically at the G_1/S border. Using an R2 promoter-luciferase reporter gene construct we demonstrate an unexpected early activation of the R2 promoter as cells pass from quiescence to proliferation. However, due to a transcriptional block, this promoter activation only results in very short R2 transcripts until cells enter the S-phase, when full-length R2 transcripts start to appear. The position for the transcriptional block was localized to a nucleotide sequence ~87 bp downstream from the first exon/intron boundary by S1 nuclease mapping of R2 transcripts from modified *in vitro* nuclear run-on experiments. These results identify blocking of transcription as a mechanism to control cell cycle regulated gene expression.

Key words: cell cycle regulation/ribonucleotide reductase/transcriptional block

Introduction

Ribonucleotide reductase is a key enzyme in the sequence of reactions leading to DNA synthesis, to which its activity is strongly correlated (Thelander and Reichard, 1979; Reichard, 1988). It catalyses the direct reduction of all four ribonucleotides to their corresponding deoxyribonucleotides.

The mammalian ribonucleotide reductase is composed of two non-identical homodimeric subunits, proteins R1 and R2, which are both essential for activity. The R1 protein levels are constant and in excess throughout the cell cycle (Engström *et al.*, 1985; Mann *et al.*, 1988) whereas the R2 protein levels are limiting and determine holoenzyme activity via an S-phase specific *de novo* synthesis followed by subsequent breakdown (Eriksson *et al.*, 1984). A sensitive RNase protection assay has been used to show that both the R1 and R2 mRNAs are specifically expressed in the S-phase both in the unperturbed cell cycle and when quiescent cells are stimulated to proliferate (Björklund *et al.*, 1990). The expression of R1 and R2 mRNA was undetectable (<2 molecules/cell) until 8 h after serum readdition when the levels increased dramatically slightly preceding the entry of cells into the S-phase.

Regulation of R1 and R2 expression at the transcriptional level has been most thoroughly studied in budding yeast, *Saccharomyces cerevisiae* and in fission yeast, *Schizosaccharomyces pombe*. In fission yeast, *cdc22*$^+$, the R1 counterpart, is unique amongst the genes encoding proteins involved in DNA synthesis, in that it is periodically expressed in late G_1 (Lowndes *et al.*, 1992). It has been shown that this regulation is conferred by binding of a protein complex that recruits the *cdc10*$^+$ gene product as one component to ACGCGT motifs in the *cdc22*$^+$ promoter. The promoters of R1 and R2 in budding yeast also contain repeats of ACGCGT motifs and furthermore are shown to be inducible by DNA damaging agents (Elledge and Davis, 1989). The promoter sequence of the mouse R2 gene shares no obvious homology to the yeast counterparts and lacks ACGCGT or related motifs (Thelander and Thelander, 1989).

Regulation of proteins taking part in DNA synthesis in mammalian cells is extensively studied and most of these enzymes show an S-phase correlated expression of their mRNA as cells are stimulated to proliferate from G_0. Though expressed specifically during the S-phase, it has not been possible to identify a common mechanism for the regulation of mRNA expression of those genes. Instead the mRNA expression for histone H4 (Lüscher *et al.*, 1985; Stauber *et al.*, 1986; Seiler-Tuynes and Patterson, 1987), thymidine kinase (Gudas *et al.*, 1988; Lipson *et al.*, 1989), dihydrofolate reductase (Leys *et al.*, 1984; Santiago *et al.*, 1984) and PCNA (Chang *et al.*, 1990) are each suggested to be regulated both at the transcriptional and post-transcriptional levels and by seemingly unrelated factors. A few studies have also suggested the importance of introns for proper regulation of some of these genes (Ottavio *et al.*, 1990; Li *et al.*, 1991; Rothender and Wintersberger, 1991) but a specific role for introns has so far not been shown.

Regulation of gene expression by transcriptional pausing or termination is thoroughly studied and plays an important role in prokaryotic organisms (for a review see Platt, 1986). In recent years, this type of regulation has also been demonstrated for a number of viral genes i.e. the transcripts initiated from the adenovirus type 2 major late promoter (Mok *et al.*, 1984), the HIV-1 long terminal repeat (Kao *et al.*, 1987) and for a few mammalian genes i.e. c-*myc*, c-*myb*, c-*fos* and adenosine deaminase (for a review see Spencer and Groudine, 1990). In common for the mammalian genes is that the transcriptional block is under metabolic or developmental control (Bentley and Groudine, 1986; Bender *et al.*, 1987; Chen *et al.*, 1990).

In this paper we report that the S-phase specific expression of mouse R2 mRNA in quiescent Balb/3T3 cells stimulated to proliferate is the result of a partial release from a transcriptional block acting in G_0/G_1 cells. To our knowledge this is the first example of a gene that shows this

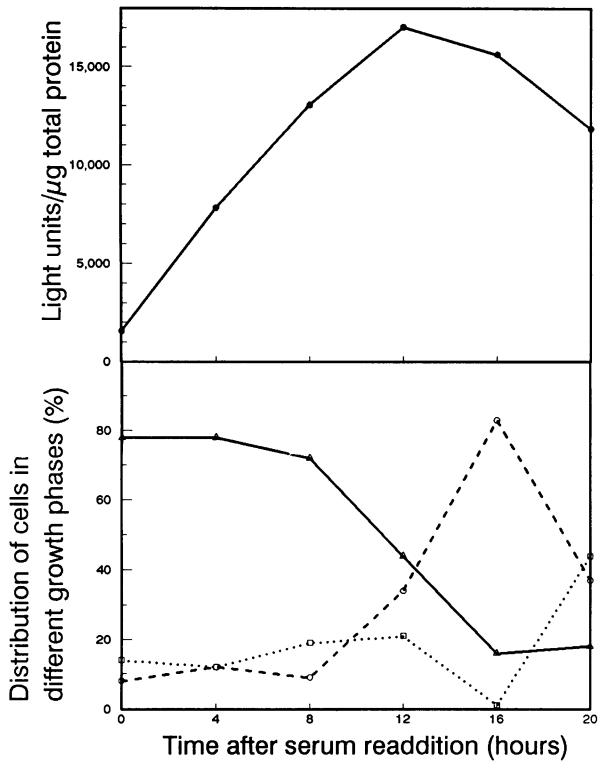

Fig. 1. (**A**) Levels of R2 promoter controlled luciferase expression in stably transformed Balb/3T3 cells synchronized by serum starvation. The ordinate gives the luciferase activity per μg of total protein. (**B**) Cell cycle phase composition, determined by DNA flow cytometry. (△) G_1-phase cells; (○) S-phase cells; (□) G_2 + M-phase cells.

type of regulation for cell cycle phase specific expression.

The position of the transcriptional block in the R2 gene was mapped by a combination of a modified *in vitro* run-on assay and an S1 nuclease protection assay.

Results

Analysis of the mouse R2 gene expression at the promoter level as cells go from a quiescent state to proliferation

To study the promoter controlled expression of the R2 mRNA we ligated a 1514 bp *Pvu*II−*Pvu*II R2 promoter fragment, containing the R2 transcription start site but not its translation start site, to the luciferase reporter gene using the vector p19luc (van Zonneveld *et al.*, 1988). This plasmid, p19lucR2 1.5, was linearized at a unique *Sca*I site in the vector, and cotransfected with the *neo* gene into Balb/3T3 cells by electroporation. Selection of stably transformed *neo* expressing cells, cloning of luciferase expressing cells and cell synchronization was performed as described in Materials and methods. Figure 1 shows the R2 promoter dependent luciferase expression in one clone at different time-points after serum readdition. Similar to earlier observed R2 mRNA levels, the R2 promoter dependent luciferase expression was low in unstimulated G_0-phase cells. However, 4 h after serum readdition, significant R2 promoter activity was detected which is much earlier than the previously observed increase in R2 mRNA. Indeed, the increase in R2 promoter dependent luciferase expression was observed already at 1 h after serum readdition (data not shown). The luciferase expression increased gradually

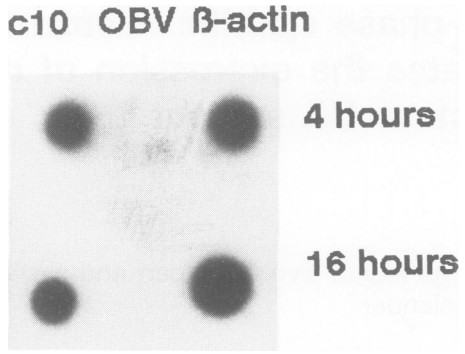

Fig. 2. Transcriptional rate at the mouse R2 gene using double stranded full-length R2 cDNA as a probe. *In vitro* elongated RNA (10×10^6 c.p.m.) prepared from Balb/3T3 nuclei harvested at the indicated time-points after serum readdition, was hybridized to double stranded DNA fixed on nitrocellulose filters. C10 is the full-length R2 cDNA cloned into the Okayama−Berg vector. OBV is the Okayama−Berg vector without insert. β-actin is the full-length rat β-actin cDNA cloned into the OBV. Distribution of cells in different cell cycle phases as measured by DNA flow cytometry was, at the 4 h time-point: G_0/G_1 = 78.5%, S = 11.3%, G_2+M = 10.2%; at the 16 h time-point: G_0/G_1 = 21.7%, S = 57.7%, G_2+M = 20.6%.

through the G_1-phase to reach a maximum in early S-phase and then declined, stabilizing at the level detected in logarithmically growing cells. Similar results were obtained with another independent clone (data not shown). The low levels of luciferase activity at the 0 h time-point are most probably contributed by cells that are not properly arrested in G_0 (see Figure 1 for distribution of cells in different growth phases). In a control experiment, Balb/3T3 cells were stably transformed with the luciferase gene linked to the Rous sarcoma virus promoter (p19lucRSV, deWet *et al.*, 1987) which does not show cell cycle dependent induction. As expected, no significant variation of luciferase expression through the cell cycle was observed using these cells (data not shown).

In vitro nuclear run-on experiments using double stranded full-length R2 cDNA as a probe

In vitro nuclear run-on transcription using nuclei from serum synchronized Balb/3T3 cells and full-length R2 cDNA as a probe was used as an independent method to study the regulation of R2 expression at the gene level. Nuclei were isolated from serum synchronized Balb/3T3 cells at 4 h (G_1-enriched cells) and 16 h (S-phase enriched cells) after serum readdition. No detectable difference in R2 transcription rate was observed between the two time-points (Figure 2). This result is in agreement with the early serum inducibility of the R2 gene expression observed using the luciferase reporter gene. However, the result is unexpected given (i) the complete absence of R2 mRNA seen in G_1-phase cells after growth stimulation, and (ii) the much later S-phase specific increase in R2 mRNA levels observed when quiescent cells are stimulated to proliferate (Björklund *et al.*, 1990).

S-phase specific expression of R2 mRNA is regulated by release from a block to transcription

In an attempt to resolve the contradictory results from the experiments with the luciferase gene, the run-on assays and the R2 mRNA measurements, we refined our *in vitro* run-on assay to allow detection of cell cycle specific regulation

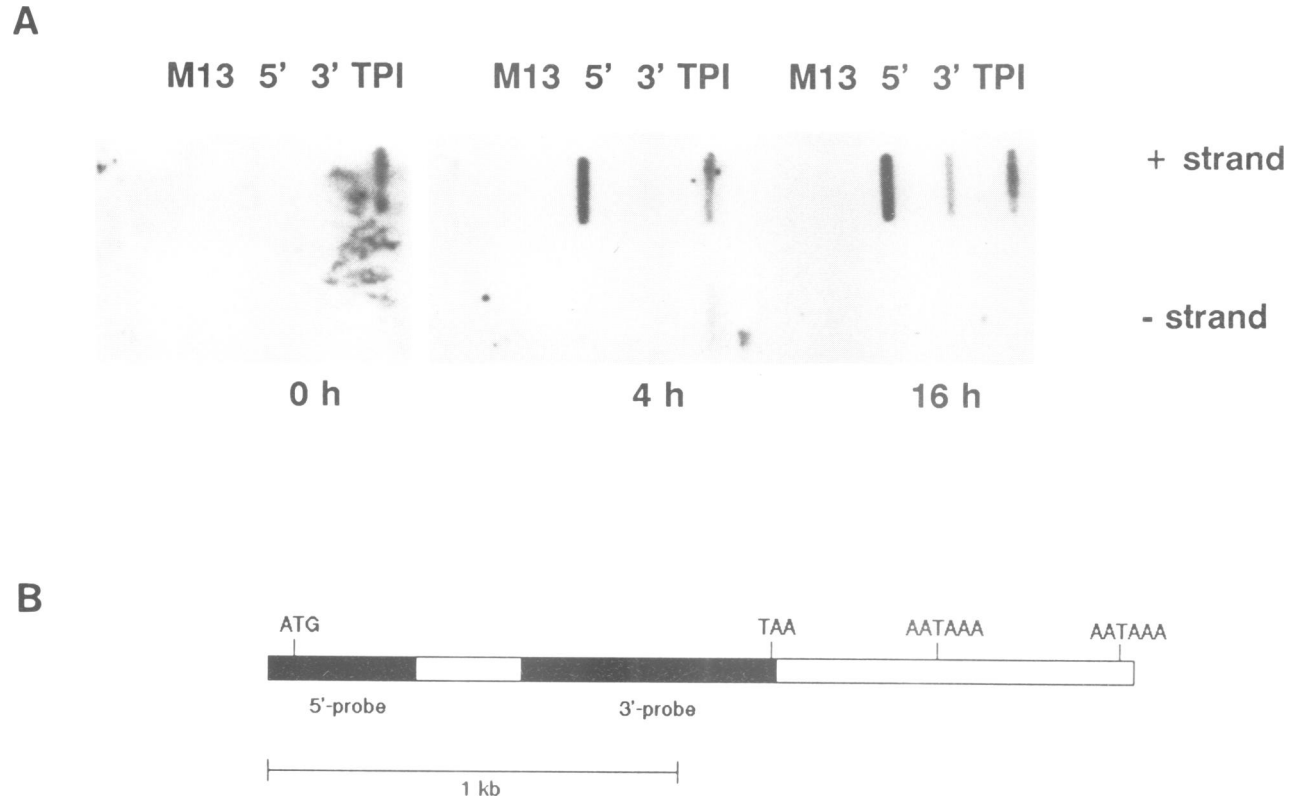

Fig. 3. Transcriptional rate at different regions of the R2 gene as quiescent Balb/3T3 cells were stimulated to proliferate. (**A**) 15×10^6 c.p.m. of *in vitro* elongated RNA prepared from Balb/3T3 nuclei at the indicated time-points were hybridized to single stranded DNA fragments blotted on nitrocellulose filters. + strand indicates detection of sense RNA by using antisense strand DNA as probes, − strand indicates detection of antisense RNA by using sense strand DNA as probes. M13 is single stranded M13mp18 DNA with no insert. 5′ and 3′ are the single stranded R2 cDNA probes described in (B) and in Materials and methods. TPI is a double stranded probe specific for triosephosphate isomerase transcripts. The Bluescript vector lacking insert gave no detectable signal in parallel experiments. The distribution of cells in different growth phases was, 0 h: G_0/G_1 = 83.2%, S = 8.4%, G_2+M = 8.4%; 4 h: G_0/G_1 = 82.6%, S = 10.1%, G_2+M = 7.3% and 16 h: G_0/G_1 = 16%, S = 80.1%, G_2+M = 3.9%. (**B**) The position for the 5′ and 3′ probes in the R2 cDNA. ATG indicates the start of R2 translation, TAA indicates where R2 translation terminates and AATAAA indicates the position for the two polyadenylation signals.

at the level of transcriptional elongation. This was achieved by using single stranded DNA fragments from different parts of the R2 gene as probes for the labelled *in vitro* run-on transcripts. Two fragments from the mouse R2 cDNA, one representing the 5′-end and the other the 3′-end of the R2 gene (for details see Materials and methods and Figure 3) were cloned into the polylinkers of the bacteriophage M13mp18 and M13mp19 to allow synthesis of both the sense and antisense strands of each fragment. The signal detected from a probe in this type of run-on transcription assay will be dependent on at least two different parameters: (i) the length of the probe; and (ii) the percentage of a certain radiolabelled nucleotide within the region of the gene complementary to each probe. The length of the R2 5′-end specific probes was 362 nt and the percentage of G residues was 25% while the 3′-end specific probes were 621 nt long and contained 27% G residues. Therefore, the 3′-end specific probe should result in a signal roughly twice as intense as the 5′-end specific probe if initiation of R2 gene transcription always resulted in full-length R2 mRNA. In each assay M13mp18 DNA without insert was used as a control for non-specific hybridization. The cDNA for triosephosphate isomerase, a housekeeping gene in the glycolytic pathway (Boyer and Maquat, 1991), was used as a positive control and for normalization of possible differences in the amount of labelled RNA added to each filter.

Figure 3 shows an autoradiogram from an experiment using the single stranded probes from the different regions of the R2 gene. There was no detectable expression of either part of the R2 gene in unstimulated, non-proliferating G_0 cells. Four hours after serum readdition, when 83% of the cells were still in G_0/G_1 according to DNA-flow cytometry, there was a high expression of the 5′-end of the R2 gene but no detectable expression of the 3′-end. At 16 h after serum stimulation, 80% of the cells were in S-phase and transcription of the entire gene was observed. However, it should be noted that even with predominantly S-phase cells, transcription of the 5′-end was much more pronounced than transcription of the 3′-end (Figure 3). No transcription of the antisense strand was detected at any point using these probes.

These results indicate that R2 mRNA expression is regulated both by an early G_1 induction of the R2 promoter and at the level of transcript elongation by a cell cycle phase specific block active in G_1 cells.

Localization of the position of the cell cycle phase specific transcriptional block in the R2 gene

The results presented above suggest that R2 mRNA expression, at least partially, is regulated by a cell cycle phase specific block to transcriptional elongation. Therefore, we were interested in developing a direct method to determine the length of R2 transcripts in cells from different phases of the cell cycle. From the results using *in vitro*

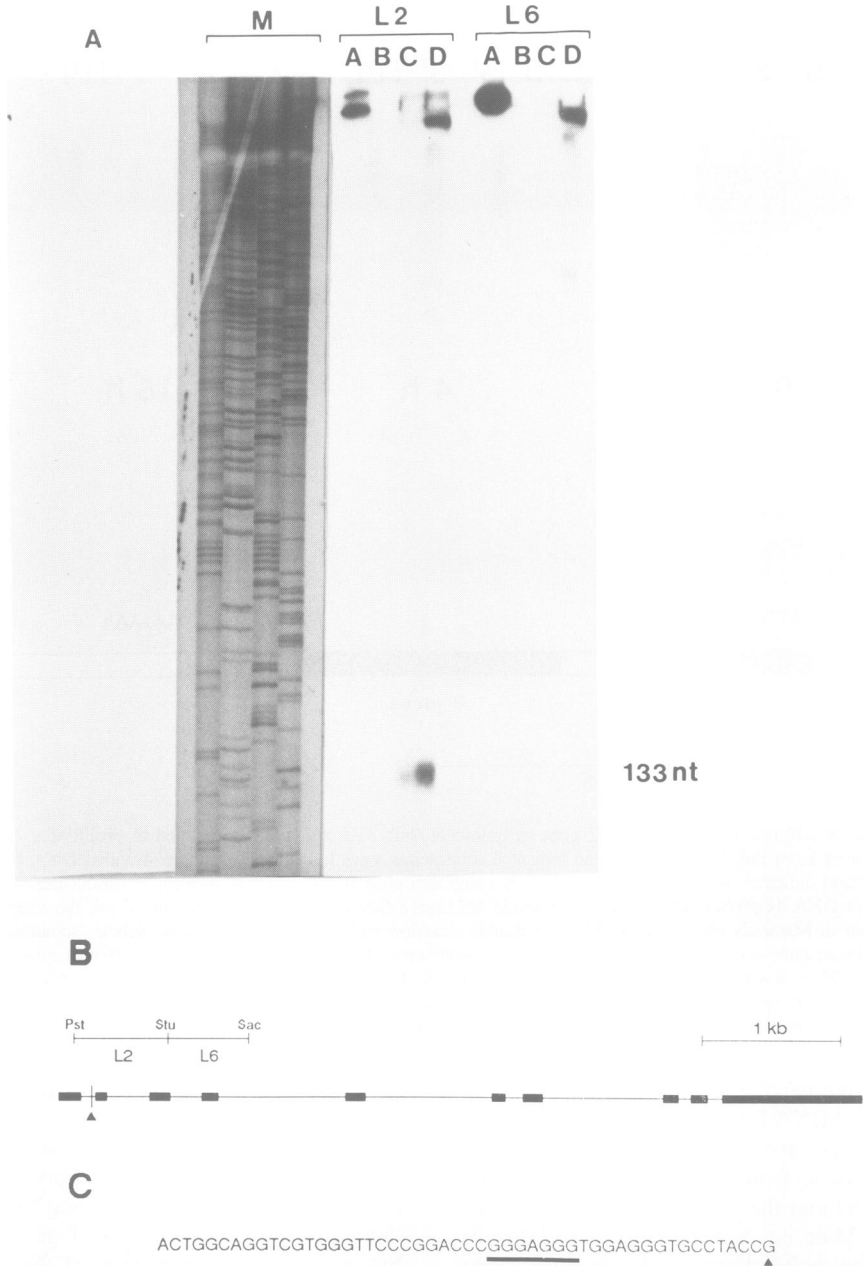

Fig. 4. Localization of the position of the cell cycle phase specific transcriptional block in the R2 gene by an S1 nuclease protection assay of *in vitro* elongated RNA. (**A**) M shows a DNA sequencing ladder of the L2 fragment (see B) where A, C, G and T, from left to right, represent the different nucleotides. L2 and L6 shows the results of S1 nuclease protection assays using the labelled L2 and L6 single stranded antisense DNA probes shown in (**B**). In these experiments, A shows the results using non-hybridized, non-digested DNA probes, B represents the results from hybridizing each probe to 50 μg of tRNA, C indicates the length of protected fragments obtained using RNA from nuclei isolated 4 h after serum readdition, and D the length of protected fragments obtained using RNA from nuclei isolated 16 h after serum readdition. The total amounts of RNA in the C and D lanes should not be compared since all the RNA recovered from each experiment (25–100 μg) was used in the hybridization. For the distribution of cells in different growth phases see the legend to Figure 3. (**B**) Position of the L2 and L6 DNA probes in the genomic R2 gene. The filled boxes represent exon sequences and the thin line represents the introns. The arrowhead indicates the transcriptional block position. (**C**) Nucleotide sequence of the proximal 50 nucleotides immediately upstream from the approximal position of the transcriptional block in the R2 gene. The arrowhead indicates the position of the transcriptional block. The consensus sequence for a potential ME1a1 binding site is underlined.

nuclear run-on assays it was obvious that the short R2 mRNAs produced were relatively stable under our assay conditions. Using this knowledge, we now isolated nuclei from cells at different phases in the cell cycle. These nuclei were then used in a modified *in vitro* nuclear run-on assay where only unlabelled ribonucleotides were added during the *in vitro* elongation step (see Materials and methods). Unlabelled, *in vitro* elongated RNA from nuclei of different cell cycle phases was then used in an S1 nuclease protection assay with labelled single stranded genomic antisense DNA probes specific for different regions of the R2 transcript. Protection of a ~133 nucleotide fragment was observed when *in vitro* elongated RNA from nuclei isolated 4 h after serum stimulation was allowed to anneal with the 663 nucleotide long L2 DNA probe, specific for the 5′-end of the R2 transcript (Figure 4). Interestingly, low amounts of the same fragment could be detected in RNA from 4 h nuclei incubated under standard run-on conditions but in the absence

of added ribonucleoside triphosphates (data not shown). This indicates that the fragment existed already *in vivo* and is not simply an *in vitro* artefact. A fragment of ~133 nt corresponds to nt +250 in the R2 gene which is 87 nt downstream from the exon 1/intron 1 boundary. In contrast, when the same probe was annealed with *in vitro* elongated RNA from nuclei isolated 16 h after serum stimulation, two fragments were protected. One corresponds to the earlier observed 133 nt fragment and the second protected fragment represents the full-length DNA probe. Using a different DNA probe, specific for the R2 gene immediately downstream from the L2 probe, we observed no protection with *in vitro* elongated RNA isolated at the 4 h time-point, confirming that this region of the R2 gene is not transcribed in G_1 cells. However, full protection of this downstream region was observed with *in vitro* elongated RNA from nuclei isolated 16 h after serum readdition.

To exclude that the observed 133 nt fragment was formed through a specific degradation of a longer transcript, a control experiment was made where [^{32}P]CMP-labelled, *in vitro* synthesized full-length R2 mRNA was incubated with nuclei under standard run-on conditions. Analysis of the RNA on a polyacrylamide−urea DNA sequencing gel showed no degradation products of discrete length but only the full-length transcript (data not shown).

Discussion

The results presented indicate that the mouse ribonucleotide reductase subunit R2 promoter is inactive in quiescent Balb/3T3 cells but it is activated very early after growth stimulation. This was demonstrated when resting Balb/3T3 cells, stably transformed with an R2 promoter−luciferase reporter gene construct, were stimulated to proliferate. The unexpected early induction of R2 gene transcription is supported by the results obtained from *in vitro* nuclear run-on experiments using full-length R2 cDNA as a probe to measure transcriptional activity in nuclei isolated from G_0/G_1-phase and S-phase enriched cells. R2 gene transcription was observed in both G_1 enriched cells and in S-phase enriched cells and surprisingly the level of transcriptional activity was similar in the two situations. Feder *et al.* also found that the rate of R2 gene transcription was similar in G_1 and S-phase cells using nuclei isolated from elutriated neonatal mice thymocytes and a double stranded cDNA probe specific for the 5′-end of the R2 gene (Feder *et al.*, 1990).

These results appear to contradict our previous finding which indicated that mouse R2 mRNA levels were undetectable in G_0/G_1 cells and did not start to increase until just before cells entered the S-phase (Björklund *et al.*, 1990). A similar expression pattern has been observed for a number of genes encoding proteins involved in DNA synthesis and the results have been interpreted as indicating post-transcriptional regulation, possibly at the level of mRNA stability (Lüscher *et al.*, 1985; Lieberman *et al.*, 1988; Chang *et al.*, 1990; Feder *et al.*, 1990).

Our results now definitively demonstrate that the delayed expression of full-length R2 mRNA transcripts results from a G_1-phase specific transcriptional block. The early induction of the R2 promoter only results in the synthesis of a short premature R2 transcript as long as cells are in the G_1-phase. This was evident from *in vitro* nuclear run-on experiments using single stranded R2 cDNA probes specific for both the 5′- and 3′-ends of the gene. S-phase specific synthesis of mature full-length R2 mRNA then occurs as a result of a cell cycle phase specific release from the transcriptional block as cells proceed into the S-phase.

It has been a problem to determine exactly the position for the transcriptional block in genes regulated by this mechanism since immature transcripts are non-polyadenylated and therefore degraded and lost when conventional methods have been used to isolate mRNA from cells or tissues (Eick and Bornkamm, 1986; Nepveu and Marcu, 1986; Bentley and Groudine, 1988). To circumvent this problem, mRNA has been isolated from *Xenopus* oocytes injected with the DNA of interest. *Xenopus* oocytes lack the mechanism for degradation of non-polyadenylated RNA (Green *et al.*, 1983) and are obviously able to synthesize both full-length and prematurely blocked transcripts but there is a clear risk of other modifications. We therefore used an S1 nuclease protection assay on unlabelled RNA prepared from a modified *in vitro* nuclear run-on transcription assay to localize directly the transcriptional block in the R2 gene to a position ~87 nucleotides downstream from the exon 1/intron 1 boundary.

There are different models for regulation of gene expression by transcriptional blocking. Modification of the RNA polymerase complex to a terminating or anti-terminating mode by binding of different cofactors is one model that is suggested for human U1 and U2 snRNAs since the termination of these genes is shown to be dependent upon their own promoters (Neuman de Vegvar *et al.*, 1986; Hernandez and Lucito, 1988). Another model is steric hindrance of the transcribing RNA polymerase by binding of transacting factors to the DNA template. This model is suggested for the human adenosine deaminase gene that exhibits premature termination when coupled to heterologous promoters and in addition shows binding of proteins to a sequence immediately downstream from the block (Chen *et al.*, 1991).

A number of reports indicate that the region immediately upstream from the block typically is capable of forming GC-rich stem-like structures in the RNA followed by a uridine stretch of at least four nucleotides thereby implicating an attenuation-like mechanism for transcriptional pausing in viral and eukaryotic genes (Hay *et al.*, 1982; Eick and Bornkamm, 1986). At the present time we cannot tell which mechanism controls the blocking of R2 transcription but the sequence upstream from the position of the transcriptional block in the R2 gene is rich in GC residues. It contains 72% GC in the 50 proximal upstream nucleotides but these residues do not seem capable of forming stem−loop structures (Figure 4).

In Figure 4 is indicated the position for a potential ME1a1 binding site which is located 17 nucleotides upstream from the position of the block. The ME1a1 protein has been proposed to induce bending of DNA and the protein is known to be important for termination of transcription of the C2 gene (Ashfield *et al.*, 1991). Potential ME1a1 binding sites can also be found downstream from the mouse adenosine deaminase transcription unit and upstream from the position of the transcriptional block in the human c-*myc* gene. Preliminary gel mobility shift assays, using an oligonucleotide corresponding to the sequence immediately upstream from the transcriptional block in the R2 gene and

a crude nuclear extract, indicate protein binding to this region (data not shown).

Regulation of mRNA expression from mammalian genes encoding proteins involved in DNA synthesis can be divided into two classes; the first class contains genes that are growth regulated and expressed in S-phase when resting cells are stimulated to proliferate but do not show any regulation in the unperturbed cell cycle. Examples of such genes are the ones encoding PCNA (Morris and Mathews, 1989), primase p49 (Tseng *et al.*, 1989), DNA polymerase α (Wahl *et al.*, 1988), thymidine kinase (Sherley and Kelly, 1988) and dihydrofolate reductase (Feder *et al.*, 1989). The second class is represented by some of the histone genes (Fletcher *et al.*, 1987; Seiler-Tuyns and Paterson, 1987; Dalton and Wells, 1988; Morris *et al.*, 1991), thymidylate synthase (Nagarajan and Johnson, 1989) and ribonucleotide reductase subunits R1 and R2 (Björklund *et al.*, 1990) which are not only growth regulated and expressed specifically in the S-phase, but also regulated in the normal cell cycle. One would expect these genes to be regulated by similar mechanisms at least within the same class, but so far comparison of the different promoter regions has indicated very few similarities.

We have now identified a block to transcription as a mechanism controlling cell cycle regulated S-phase specific expression of the R2 subunit of mouse ribonucleotide reductase. This mechanism may also regulate the cell cycle specific expression of other genes within the second class.

Materials and methods

Cell culture and cell synchronization

Balb/3T3 cells (ATCC CCL 163) were grown as monolayer cultures in Dulbecco's Modified Eagle's Medium (DMEM) supplemented by 10% heat-inactivated horse serum (HIHS) as described earlier (Björklund *et al.*, 1990). For serum synchronization cells were plated at a density of 2×10^6 cells per 14 cm dish, or 1×10^6 per 8 cm dish. After 48 h, cells were washed once with serum-free medium and 25 ml of medium containing 0.6% fetal calf serum (FCS) were added. After 48 h, this medium was replaced by a medium containing 20% FCS, and cells were subsequently harvested and handled as described for each method. At each time-point, aliquots of cells (0.5×10^6) were removed for fixation in 96% ethanol and subsequent flow cytometry analysis of DNA content (Eriksson *et al.*, 1984).

Cell transfection and selection of stable transformants

Balb/3T3 cells were plated at a density of 1×10^6 cells per 14 cm dish and incubated at 37°C for 48 h. Cells were harvested by trypsinization at ~50% confluency, washed once with 1× phosphate-buffered saline and suspended in serum-free DMEM at a concentration of 7×10^6 cells per ml. 0.8 ml of cell suspension was mixed with 18 μg of linearized reporter gene DNA and 2 μg of pSV2neo (Southern and Berg, 1982) and incubated on ice for 10 min. Electroporation was performed in cuvettes with 0.2 μm electrode gap using a Bio-Rad gene pulser set at 270 V and 960 μF as described (Chu *et al.*, 1987). After electroporation, the cell suspension was incubated on ice for 10 min and then cells were plated in 25 ml DMEM + 10% HIHS. Selection of neo-resistant cells was initiated 48 h after seeding by adding 550 μg/ml of Geneticin base (Sigma) to fresh DMEM + 10% HIHS. Medium and drug were changed every second day until well separated neo-resistant clones were visible. Individual clones were expanded, tested for luciferase activity and frozen in liquid nitrogen.

Plasmid constructs

The plasmid p19lucR2 1.5 used as an R2 reporter gene was constructed by ligation of a *PvuII*–*PvuII* DNA fragment from the R2 promoter (nt −1497 to nt +17 according to Thelander and Thelander, 1989 where +1 indicates the position for the start of R2 gene transcription) to a unique *SmaI* in the polylinker of the vector p19luc (van Zonneveld *et al.*, *1988*). M13R2.2 (5′-sense), M13R2.1 (5′-antisense), M13R213.1 (3′-sense) and M13R213.5 (3′-antisense) were used to detect labelled transcripts from *in vitro* run-on experiments. M13R2.2 and M13R2.1 were constructed by ligation of a *StuI* (present in the Okayama–Berg vector) to *TaqI* [nt +319 fragment from the mouse R2 cDNA (Thelander and Berg, 1986)] into the polylinkers of M13mp18 and mp19. M13R213.1 and M13R213.5 were constructed by ligation of a *Sau*3a–*Sau*3a DNA fragment (nt +620 to +1241 in the mouse R2 cDNA) into M13mp18 and they are oriented in the opposite directions. pTPI was a gift from Dr Martin Gullberg and consists of a 630 nt *SacI*–*EcoRI* DNA fragment from the cDNA for triosephosphate isomerase (Maquat *et al.*, 1985) ligated into the Bluescript vector polylinker. pSV2 neo was previously described by Southern and Berg (1982). The L2 DNA probe is a 663 nt long *PstI* (nt +250)–*StuI* (nt +913) fragment from the R2 genomic gene (Thelander and Thelander, 1989) cloned into M13mp19 and L6 is a 569 nt *StuI* (nt +913)–*SstI* (nt +1484) DNA fragment cloned into M13mp19. Labelled full-length R2 mRNA was synthesized *in vitro* as described (Björklund *et al.*, 1990) using a *PvuII* (nt +17)–*XhoI* [present in the Okayama–Berg vector downstream from the 3′-end of the R2 cDNA (Thelander and Berg, 1986)] DNA fragment ligated into the polylinker of pGEM-3 (Promega).

Luciferase assays

Harvesting of cells and preparation of cell extracts for luciferase assays were performed exactly as described (deWet *et al.*, 1987). Measurements of luciferase activity were performed using a Berthold 9500 T luminometer equipped with a chart recorder and an automatic injector. The luciferase activity in cell extracts was measured as the number of light units emitted during a 10 s period per μg of total protein, determined by the Bradford protein assay (Bradford, 1976).

In vitro nuclear run-on

Preparation of Balb/3T3 nuclei from cells in different growth phases was performed essentially as described (Groudine *et al.*, 1981) with the following modifications. Balb/3T3 cells were synchronized as described and from each time-point $0.5-1\times10^8$ cells were harvested and dissolved in 1 ml NP-40 lysis buffer (10 mM Tris–HCl pH 7.4, 3 mM $CaCl_2$, 2 mM $MgCl_2$ and 0.5% Nonidet P-40) per 5×10^7 cells. Cells were incubated on ice for 5 min and transferred to an ice-cold Dounce homogenizer and homogenized by 5–10 strokes using a loose pestle until >90% of the cells were lysed as determined by phase-contrast microscopy. Nuclei were collected by centrifugation (500 *g*, +4°C for 5 min), washed twice in buffer A (40 mM Tris–HCl pH 7.9, 20% glycerol, 240 mM KCl, 10 mM $MgCl_2$, 0.1 mM EDTA and 1 mM 2-mercaptoethanol) and suspended in 0.1 ml buffer A containing 40% glycerol and frozen at −70°C. *In vitro* elongation of transcripts was performed exactly as described by Chen *et al.* (1990).

Linearized plasmid (10 μg) or circular single stranded (5 μg) DNA probes were denatured in 0.4 N NaOH and dot blotted on to nitrocellulose membrane filters (Amersham) through a Bio-Rad apparatus as recommended by the manufacturer. Prehybridization, hybridization and washing were performed as described by Chen *et al.* (1990). Labelled nascent transcripts from the same number of cells corresponding to an equal number of c.p.m. ($10-20\times10^6$ c.p.m.) were added to each filter. After washing the filters were exposed to XAR-5 film (Kodak) in −70°C for up to 10 days.

Determination of the length of prematurely terminated transcripts

Transcripts were elongated *in vitro* by using nuclei prepared from Balb/3T3 cells at different time-points after growth stimulation as described above but with the following modifications. No labelled ribonucleotide was added to the elongation step and the concentration of GTP was increased to 0.6 mM. The partial hydrolysis step using 0.2 N NaOH, normally used to shorten the labelled nascent transcripts, was omitted and *in vitro* elongated RNA was instead immediately ethanol precipitated after the proteinase K digestion and phenol/chloroform extraction. Antisense DNA probes, L2 and L6, specific for different regions in the 5′-end of the R2 gene were uniformly labelled by filling in reactions using [α-^{32}P]dCTP, the Klenow fragment of DNA polymerase I, the 17mer M13 sequencing primer and a sense oriented DNA template (Maniatis *et al.*, 1982). Single stranded probes were isolated after endonuclease digestion and separation on denaturating polyacrylamide–urea gels followed by electroelution. The specific activity of labelled fragments was typically 4×10^7 c.p.m./μg DNA. 50×10^3 c.p.m. of labelled antisense DNA probes were mixed with 25–100 μg *in vitro* elongated RNA and ethanol precipitated. After centrifugation the pellet was resuspended in 10 μl hybridization buffer (80% deionized formamide, 400 mM NaCl, 40 mM PIPES pH 6.4 and 1 mM EDTA), denatured at 85°C for 5 min and hybridized at 53°C for 4 h. After hybridization, S1 nuclease digestion was performed using 0.2 ml of a solution containing 280 mM NaCl, 30 mM NaAc pH 4.4, 4.5 mM $ZnCl_2$, 20 μg/ml heat-denatured calf-thymus DNA and 350 U/ml of S1 nuclease (Pharmacia) at 37°C for 30 min. Reactions were terminated by the addition of 30 μl of stop buffer (4 M ammonium acetate, 1% SDS and 400 μg/ml tRNA). Protected fragments

were ethanol precipitated and separated on 6% polyacrylamide–8 M urea DNA sequencing gels.

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