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Transcriptional activity of the human thymidine kinase gene determined by a method using the polymerase chain reaction and an intron-specific probe

(reverse transcription/WI-38 diploid fibroblasts/serum stimulation/cell cycle/mRNA precursor)

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ABSTRACT **We have used the technique of reverse transcription coupled to the polymerase chain reaction to detect mRNA precursors [heterogeneous nuclear RNA (hnRNA)] transcribed from the thymidine kinase (TK) gene of human diploid fibroblasts. With this method, the amplification products of both hnRNA (containing the introns) and mature mRNA can be detected on Southern blots with appropriate hybridization probes. With the experimental conditions used, the sensitivity of the technique is such that TK mRNA can be detected in as few as 20 S-phase cells. TK hnRNA is maximally expressed early in the S phase of the cell cycle after quiescent human fibroblasts are stimulated to proliferate. At this point, the ratio of TK hnRNA to TK mRNA is 1:155. A small amount of TK hnRNA can be detected in populations of cells that appear to be quiescent. However, the presence of the precursor in these populations correlates with the number of cells still cycling. No TK hnRNA can be detected in truly quiescent human diploid fibroblasts, suggesting that in these cells, the TK gene is not transcribed in G_0.**

Regulation of the expression of the thymidine kinase (TK) gene is complex and occurs at transcriptional, posttranscriptional, and translational levels (1–5). The primary mode of regulation varies considerably between cell types. For example, posttranscriptional/translational regulation of enzyme activity predominates during myoblast differentiation and in continuously cycling HeLa cells (6, 7). In contrast, the activity of TK, the amount of the enzyme, and the steady-state levels of TK mRNA are very low or undetectable in nonproliferating, G_0 human or mouse fibroblasts, and all increase sharply after stimulation as the cells enter the S phase of the cell cycle (1, 8, 9). This difference between G_0 cells stimulated to proliferate and exponentially growing cells is not unique to the TK gene. DNA polymerase α is also constitutively expressed in cycling cells but is inducible by growth factors in G_0 cells (10).

We (3, 11) and others (4) have demonstrated that the 5′ flanking sequence of the human TK gene is important for the S-phase-specific expression of its mRNA. For example, Kim *et al.* (4) have shown that TK 5′ flanking sequences can confer S-phase specificity on a heterologous promoter and reporter gene. This indicates that the transcriptional activity of the TK gene is modulated in a cell cycle-dependent manner since 5′ flanking sequences mediate transcriptional regulation of gene expression. Run-on transcription assays (12, 13) indicate that the transcriptional activity of the TK gene increases transiently by 4- to 6-fold as cells enter S phase (1, 2). However, they also suggest that TK is transcribed in serum-deprived mouse fibroblasts (1, 2).

In this investigation, we have examined the steady-state amount of TK mRNA precursor [TK heterogeneous nuclear RNA (hnRNA)] by a method that uses reverse transcription coupled to the polymerase chain reaction (RT–PCR) (14, 15). The amount of hnRNA depends on the rate of splicing as well as transcription, and therefore RT–PCR provides only indirect information on the transcriptional activity of the gene. However, the ability to amplify the signal with the PCR is a significant advantage that can consistently give reliable results for low-abundance messages by increasing the signal-to-noise ratio. The results show that no TK hnRNA can be detected by this sensitive method when human diploid fibroblasts (WI-38 cells) are truly quiescent.

EXPERIMENTAL PROCEDURES

Cell Culture. WI-38 normal human diploid fibroblasts were grown as described (8). WI-38 cells were made quiescent by plating at a density of 2×10^4 per cm^2 followed by incubation for 8 or more days (as indicated) without a medium change. Quiescent cells were stimulated by adding fresh growth medium.

RNA Isolation. Total RNA was isolated by the method of Chomczynski and Sacchi (16). To ensure that no DNA was present, the RNA was centrifuged through a cushion of 5.7 M CsCl (17). For this, the RNA was thoroughly dissolved in 25 μl of denaturing solution [4 M guanidinium thiocyanate/25 mM sodium citrate, pH 7.0/0.5% sarkosyl (*N*-lauroylsarcosine)/0.1 M 2-mercaptoethanol] and then 25 μl of 5.7 M CsCl/0.1 M EDTA, pH 8, was added. This was layered over a cushion of the CsCl/EDTA solution (100 μl) in a 5 × 20-mm cellulose propionate tube and centrifuged in a Beckman Airfuge for 1.75 hr at 100,000 × *g*. The liquid was removed and the pellet was dissolved in diethyl pyrocarbonate-treated water, adjusted to 1 M LiCl, and precipitated with 7 volumes of ethanol. The RNA was dissolved in diethyl pyrocarbonate-treated water and visually checked for integrity and the absence of DNA by electrophoresis in a nondenaturing agarose gel containing ethidium bromide. When DNA was present, it could be detected as a fluorescent band at the well. By this technique, we could rule out any DNA contamination. The results also confirmed the absence of DNA, since no amplification signal was detectable in truly quiescent WI-38 cells (see below).

RT–PCR. The procedure for reverse transcription and PCR amplification was similar to published methods (14, 15, 18). One microgram (5 μl) of RNA was transferred to a 0.5-ml microcentrifuge tube and the following were added: 5× reverse transcriptase buffer (4 μl, BRL), 3′ primer (1 μl, 100 ng), and a mixture of 2.5 mM dNTPs (8 μl). The tube was put

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Abbreviations: PCR, polymerase chain reaction; RT–PCR, reverse transcription coupled with PCR; TK, thymidine kinase; hnRNA, mRNA precursor(s) (heterogeneous nuclear RNA).

in a boiling water bath for 2 min and then the entire bath was transferred to a 55°C water bath for 30 min. RNasin (1 μl, 25–40 units, Promega) and Moloney murine leukemia virus reverse transcriptase (2 μl, 400 units, BRL) were added to the mixture, which was then incubated at 39°C for 60 min. After reverse transcription, water (46 μl), more 3′ primer (5 μl, 500 ng), 5′ primer (6 μl, 600 ng) and 10× AmpliTaq buffer (10 μl, Perkin–Elmer/Cetus) were added. The tube was incubated in a boiling waterbath for 5 min and then at 55°C for 5 min. Nucleotides (12 μl, 1.25 mM each dNTP) and *Thermus aquaticus* DNA polymerase (AmpliTaq, 1 μl, 5 units) were added and the mixture was covered with a layer of mineral oil. After 2 min of incubation at 55°C and 3 min at 70°C, the samples were transferred to a Perkin–Elmer/Cetus Thermal Cycler for 40 cycles of PCR. Each cycle consisted of 2 min at 94°C, 3 min at 55°C, and 4 min at 70°C with 1-min transitions. The results of RT–PCR were reproducible in repeated experiments.

Southern Blotting. Southern blotting (19) was done by standard procedures on a 4% NuSieve GTG agarose (FMC) gel. After denaturation, the gel was neutralized in 1 M ammonium acetate and the DNA was transferred by capillary action to Zetabind (AMF Cuno) with 1 M ammonium acetate for ≈18 hr. Transferred DNA was attached to the filter by UV crosslinking.

Probes. The intron-specific oligonucleotide probe (see Fig. 1) was labeled with polynucleotide kinase and [γ-^{32}P]ATP (20) to a specific activity of 7.5×10^8–2.1×10^9 dpm/μg. The probe used to detect exon sequences was an oligonucleotide (AGTGGGCAGGTTAATGC) complementary to a sequence in the first exon, labeled with polynucleotide kinase (specific activity, 5.4×10^8 dpm/μg), or a *Pst* I–*Taq* I fragment of TK cDNA from pTK11 (21) labeled by the random priming method (22) (specific activity, 8×10^8 dpm/μg).

RESULTS

In this investigation we examined the amount of human TK hnRNA in WI-38 human diploid fibroblasts, which are exquisitely growth-regulated and can be made quiescent by contact inhibition. To detect TK hnRNA in quiescent cells and in cells stimulated to proliferate, we used RT–PCR. This technique involves reverse-transcribing RNA and then amplifying the resultant RNA·DNA hybrids by the PCR (14, 15). We reasoned that if we were to reverse-transcribe total RNA with a primer in the second exon of TK (Fig. 1) and then amplify with another primer in the first exon, the products should include one that contains the first intron. This species could then be detected with an intron-specific probe on a Southern blot of the amplification products.

Since TK mRNA is not an abundant species and its precursor should be even less abundant, it was necessary to first determine the limit of detectability of TK hnRNA. For this purpose, quiescent WI-38 human fibroblasts were stimulated with serum for 16 hr and then decreasing numbers of these cells were mixed with BALB/c mouse 3T3 fibroblasts to give a total of 1.1×10^6 cells. Total RNA from the cell mixtures was then subjected to the RT–PCR process using amplification primers specific for human TK. A Southern blot of the amplification products was probed with an oligonucleotide complementary to a sequence in the first exon (Fig. 2*A*), which simultaneously identified the amplification products of human TK hnRNA (247 bp) and mRNA (136 bp), or with an intron-specific probe (Fig. 2*B*). mRNA was detectable when 1000 WI-38 cells were present in the cell mixture. With the same exon probe, hnRNA was detectable only when at least 100,000 WI-38 cells were used (Fig. 2*A*). However, these are not the limits of detection. In an overexposed autoradiograph, the amplification product of TK mRNA could be detected when 100 WI-38 cells were diluted with 1.1×10^6 BALB/c 3T3 cells (data not shown). Furthermore, when the intron probe was used to avoid interference from the mRNA signal, TK hnRNA was clearly visible in the sample representing 10,000 WI-38 cells (Fig. 2*B*). In this experiment, the number of WI-38 cells in S phase after serum stimulation was determined by autoradiography with [^{3}H]thymidine to be 20%. Thus, the limits of detection under our experimental conditions are ≈2000 cells in S phase for TK hnRNA (intron probe) and 20 cells for the mature mRNA.

The detection limits suggest that the amount of TK mRNA precursor represents 0.1–1% of TK mRNA 16 hr after serum stimulation. Densitometric comparison of an autoradiograph probed with the exon-specific oligonucleotide indicated that there was ≈15.5 times more TK mRNA in the sample containing 10,000 WI-38 cells than TK hnRNA in the sample containing 100,000 WI-38 cells. This indicates that 16 hr after serum stimulation, the ratio of TK mRNA to TK hnRNA is about 155:1.

Having established that an amplification product of TK hnRNA could be detected by RT–PCR from as few as 20,000 S-phase cells, we investigated the abundance of the TK hnRNA under various growth conditions. Quiescent WI-38 fibroblasts were stimulated with serum for various times and RNA from these cells was used for RT–PCR. A Southern blot probed with the intron-specific oligonucleotide (Fig. 3) re-

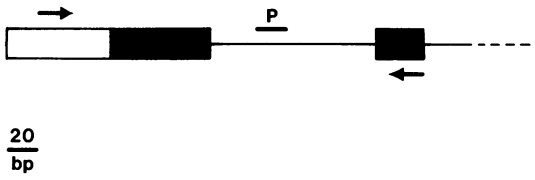

FIG. 1. Structure of the 5′ end of the human TK hnRNA. The exons are shown as boxes, filled if they represent coding sequences. Thin lines indicate introns. The location of the 5′ amplification primer (CTTGGAGAGTACTCGGGTTCGTG) is shown as a rightward arrow above the first exon. The location of the 3′ primer (CTTTTTCCTGAGAACATCGGC) used for reverse transcription and PCR amplification is shown as a leftward arrow below the second exon. The probe used for detection of amplification products containing the intron (ATGAGGTGGTCGTGGTGATAG) is shown as a bar above the first intron and labeled P. The sequences for the amplification primers and the intron probe were derived from the genomic sequence submitted to GenBank by Flemington *et al.* (23). bp, Base pairs.

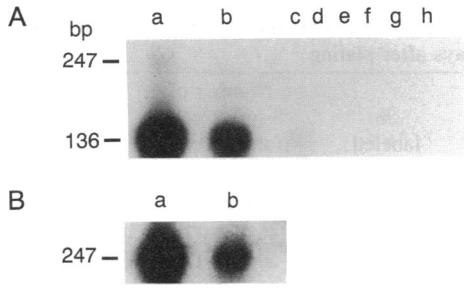

FIG. 2. Limits of detection of TK hnRNA and comparison of its abundance with TK mRNA. Eight days after plating, confluent WI-38 human fibroblasts in a 100-mm tissue culture dish were stimulated with 10% fetal bovine serum for 16 hr. The harvested cells were mixed in decreasing numbers with exponentially growing BALB/c 3T3 cells to a total of 1.1×10^6 cells. Total RNA was isolated from the cell mixtures and subjected to the RT–PCR procedure. Southern blots of the amplification products were probed with an oligonucleotide specific for exon 1 (*A*) or with an intron-specific oligonucleotide (*B*) (see *Experimental Procedures*). The autoradiograph shown in *A* (exon probe) was exposed for 5 hr without an enhancing screen, while that in *B* (intron probe) was exposed for 72 hr with a Cronex Lightning Plus enhancing screen (DuPont). The number of WI-38 cells in each sample was as follows: lane a, 100,000; lane b, 10,000; lane c, 1000; lane d, 100; lane e, 10; lane f, 1; lane g, 1; lane h, 0.

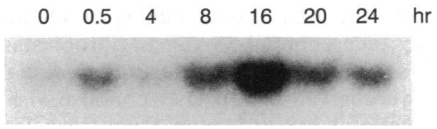

FIG. 3. TK hnRNA in serum-stimulated WI-38 human diploid fibroblasts. WI-38 cells were plated at a density of 2×10^4 cells per cm^2 in 100-mm tissue culture dishes in growth medium containing 10% fetal bovine serum. Eight days after plating, the cells were stimulated with fresh medium containing 10% fetal bovine serum and RNA was isolated after various times (shown in hours above each lane). The RNA was subjected to RT–PCR and the resultant blot was hybridized with the oligonucleotide probe specific for the first intron of the human TK gene.

vealed the presence of small amounts of TK hnRNA in cells that had not yet been stimulated with serum. A small transient increase in TK hnRNA was observed 30 min after serum stimulation, but the significance of this is undetermined. The amount of TK hnRNA increased by 8 hr after addition of serum (when the cells were still in G_1), reached a maximum at 16 hr, and then began to decrease. The expression of TK hnRNA at 16 hr was >10-fold greater than in G_0 cells, indicating a large increase in the number of TK transcripts coincident with entry into S phase (24, 25). These results were reproducible.

The presence of TK hnRNA in quiescent cells could indicate that cells in G_0 transcribe the TK gene at a low level. However, it is known that when WI-38 cells are plated in growth medium and left alone for 8 days, a few cells will still be traversing the cell cycle (26). Therefore, an alternative explanation for detecting TK hnRNA before serum stimulation is that the number of cells that were in S phase exceeded the detection limits of the assay. To test this, we used an experiment that was originally done by Augenlicht and Baserga in 1974 (26) and has subsequently been repeated with other cell types including WI-38 cells (27, 28). Cells were plated in growth medium and labeled with [^{3}H]thymidine for 24 hr on various days after plating to determine the percentage of cells in S phase (Table 1). The fraction of cells that continued to incorporate [^{3}H]thymidine decreased to a few percent by the 5th day after plating. However, proliferating cells were still detectable 8 and 12 days after plating and were undetectable only after 20 days.

Table 1. Fraction of cells that incorporate [^{3}H]thymidine on various days after plating

Day	% labeled	Number of S-phase cells (fold increase)	TK hnRNA, densitometer units (fold increase)
2	40	400,000 (20)	85 (21)
5	1.8	30,000 (1.5)	8.5 (2.0)
8	2.8	46,670 (2.3)	9.0 (2.3)
12	3.0	50,000 (2.5)	8.5 (2.1)
20	0	0	—
Control	20	20,000 (1)	4.0 (1)

WI-38 cells were plated at a density of 2×10^4 per cm^2 in growth medium containing 10% fetal bovine serum in 35-mm tissue culture dishes containing coverslips. On the indicated days, [^{3}H]thymidine was added to duplicate cultures at a final concentration of 0.1 μCi/ml (1 μCi = 37 kBq). After 24 hr, the medium was removed and the coverslips were washed three times with Hanks' balanced salts solution and then fixed for 30 min with −20°C methanol. After 2 days of autoradiography, ≈1500 cells were counted per coverslip. The fraction of cells labeled by [^{3}H]thymidine is listed above. The control sample represents cells that were stimulated with serum for 24 hr 8 days after they were plated. The number of S-phase cells was calculated by assuming 5×10^6 cells per confluent 100-mm culture (3×10^6 cells on day 2). Arbitrary densitometry units were derived from Fig. 4 with a Zeineh laser densitometer. The fold increase over the control sample is shown in parentheses.

In parallel, RNA was isolated on various days after plating and subjected to RT–PCR (Fig. 4*A*). TK hnRNA could be detected up to the 12th day after plating, but disappeared by the 20th. The Southern blot in Fig. 4*A* was probed with the intron-specific oligonucleotide and purposely overexposed (with respect to Fig. 3) in order to show that no amplification signal is detectable in cells 20 days after plating. TK mRNA was also detectable on the 12th but not the 20th day after plating (data not shown). It should be noted that if these cells are restimulated with serum, they will respond with DNA synthesis and proliferation (24–27). WI-38 cells reach confluence on about the 5th day after plating at a density of ≈5×10^6 cells per 100-mm dish, and the number of cells does not appreciably increase thereafter (26). Since the number of S-phase cells was known for the sample shown in Fig. 2, lane a, RNA from that experiment was processed in parallel (Fig. 4*B*) to provide an estimate of the amount of TK hnRNA present after 8 or 12 days. Densitometry of an underexposed autoradiograph demonstrated that the intensity of the bands correlated with the fraction of cells synthesizing DNA (Table 1). Two to three times more TK hnRNA was present in 5×10^6 WI-38 cells 8 days after plating than in the RNA sample containing 20,000 cells in S phase. Since ≈3% of the cells (150,000) were labeled by [^{3}H]thymidine during a 24-hr period on the 8th day (Table 1), and since S phase in WI-38 cells lasts about 8 hr, 50,000 cells would be expected to be in S at the time of RNA isolation. This is 2.5-fold greater than the 20,000 cells calculated for the control sample (Fig. 4*B*) and is remarkably similar to the densitometric quantitation (Table 1). Therefore, the TK hnRNA detected in unstimulated, quiescent cells 8–12 days after plating was probably due to cells expressing TK RNA at normal levels in S phase rather than at low levels constitutively.

All of the experiments described above were done with WI-38 cells. To test the technique in another system, we examined TK hnRNA expression in HL-60 promyelocytic leukemia cells induced to differentiate with phorbol 12-myristate 13-acetate (29). RNA was isolated from exponentially growing HL-60 cells or from HL-60 cells exposed to the phorbol ester for 60 hr. After RT–PCR, the amplification product of TK hnRNA was detected easily in HL-60 RNA and diminished after treatment with phorbol ester (data not shown).

DISCUSSION

Since mRNA precursors (hnRNA) are transient intermediates, their concentration at any given time reflects their rates of formation (transcription) and processing (splicing). There-

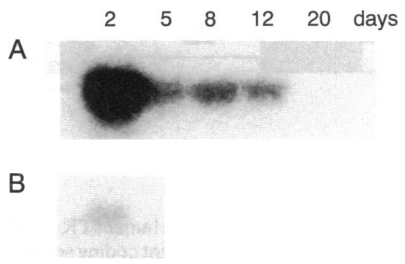

FIG. 4. TK hnRNA in quiescent WI-38 fibroblasts. (*A*) WI-38 cells were plated as described in Fig. 3 and RNA was isolated after various times (shown in days above each lane). The Southern blot of amplification products of the RT–PCR procedure was hybridized with the intron probe. The percentage of cells labeled with [^{3}H]thymidine in a 24-hr period in the same experiment is shown in Table 1. (*B*) RNA from 20,000 S-phase WI-38 cells (Fig. 2, lane a) was simultaneously subjected to the RT–PCR procedure to facilitate quantitation of TK hnRNA.

fore, increases in hnRNA reflect increases in transcription and/or decreases in the rate of processing of the hnRNA to mature mRNA. Thus, RT–PCR detection of hnRNA provides information on transcriptional activity indirectly by measuring the relative amount of a transient intermediate that is produced by transcription. Because of the increased sensitivity afforded by the PCR, our method is valid only if DNA is rigorously excluded. To accomplish this, we have purified total RNA by centrifugation through a CsCl cushion (17). The reproducible variation in intensity of the signals in Figs. 3 and 4 and its correlation with the number of cells in S phase as well as the absence of a detectable signal in truly quiescent cells are the best demonstration that hnRNA and not DNA was detected, since RNA was isolated from the same number of cells and equal amounts (1 μg) were subjected to RT–PCR.

Transcriptional activity is usually determined with the run-on transcription assay, which directly measures incorporation of nucleotides into nascent RNA of genes in the process of being transcribed (12, 13). RT–PCR detection of hnRNA measures something different: the steady-state level of mRNA precursors at any given moment. This is analogous to the assay of steady-state levels of mature mRNA by RNA blotting. It will therefore complement, rather than replace, run-on transcription assays. RT–PCR is particularly useful when detection of run-on transcripts is difficult because the gene to be studied is expressed at low levels, as the TK gene is.

The primary advantage of this technique is the ability to amplify a signal that would otherwise be undetectable. A second attractive feature of the method is that formation of the hnRNA that is being measured occurs *in vivo* under physiological conditions. A third advantage is that RT–PCR can be done with a much smaller number of cells than is necessary for run-on transcription. For example, one 100-mm dish of WI-38 cells is sufficient for RT–PCR, whereas 20–30 plates would be needed for run-on transcription. When the experiment involves several time points as in the present data, the difference comes to 7 vs. 200 plates. Because of this sensitivity, RT–PCR could also be quite useful for determining transcriptional activity in transfected cell cultures (transient-expression assays) in which the fraction of transfected cells is usually low. For such transient-expression assays, it may be necessary to treat the RNA with DNase if centrifugation through a CsCl cushion is not sufficiently rigorous to completely exclude transfected plasmid DNA.

As already stated, because RT–PCR derives information indirectly, it will not replace the run-on transcription assay. However, we believe that the RT–PCR method of detecting hnRNA is a technique with general applicability that can add significant information about the transcriptional activity of cells.

The following conclusions were derived from using this technique to examine the transcriptional regulation of human TK in diploid fibroblasts. (*i*) In WI-38 cells, TK hnRNA can be detected with 2000 S-phase cells under the conditions used for these experiments. The amount of TK hnRNA is 0.1–1% of TK mRNA at its maximal concentration. (*ii*) Truly quiescent WI-38 cells do not have detectable amounts of TK hnRNA. Both hnRNA and mature mRNA can be detected 12 days after plating, when the cells are generally considered quiescent (24, 25, 27, 28). However, the amount of hnRNA detected in these cells can be attributed to the small number of cells that are still cycling. The fact that no TK mRNA is detectable in truly quiescent WI-38 cells (20 days after plating) suggests that the absence of TK hnRNA is not due to increased processing but to the absence of transcription. (*iii*) The amount of TK hnRNA increases transiently as serum-stimulated fibroblasts enter S phase of the cell cycle. This indicates that the transcriptional activity of the TK gene increases at least 10-fold. However, the exact transcription rate cannot be accurately estimated since splicing efficiency is also enhanced at this time (5). (*iv*) The RT–PCR method of detecting hnRNA can be used to investigate the transcriptional activity of genes. It is particularly useful for genes with weak promoters that produce low-abundance RNAs that can be difficult to detect by the run-on transcription assay and in transient-expression assays of transfected cells. Its sensitivity results in dramatic decreases in the number of cultured cells needed for the experiment.

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1. Coppock, D. L. & Pardee, A. B. (1987) *Mol. Cell. Biol.* **7**, 2925–2932.
2. Stewart, C. J., Ito, M. & Conrad, S. E. (1987) *Mol. Cell. Biol.* **7**, 1156–1163.
3. Travali, S., Lipson, K. E., Jaskulski, D., Lauret, E. & Baserga, R. (1988) *Mol. Cell. Biol.* **8**, 1551–1557.
4. Kim, Y. K., Wells, S., Lau, Y.-F. C. & Lee, A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 5894–5898.
5. Gudas, J. M., Knight, G. B. & Pardee, A. B. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 4705–4709.
6. Sherley, J. L. & Kelly, T. J. (1988) *J. Biol. Chem.* **263**, 8350–8358.
7. Gross, M. K. & Merrill, G. F. (1988) *Nucleic Acids Res.* **16**, 11625–11643.
8. Rittling, S. R., Brooks, K. M., Cristofalo, V. J. & Baserga, R. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 3316–3320.
9. Chang, Z.-F. & Chen, K. Y. (1988) *J. Biol. Chem.* **263**, 11431–11435.
10. Wahl, E. F., Geis, A. M., Spain, B. H., Wong, S. W., Korn, D. & Wang, T. S. F. (1988) *Mol. Cell. Biol.* **8**, 5016–5025.
11. Lipson, K. E., Chen, S.-T., Koniecki, J., Ku, D.-H. & Baserga, R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 6848–6852.
12. Groudine, M., Peretz, M. & Weintraub, H. (1981) *Mol. Cell. Biol.* **1**, 281–288.
13. Greenberg, M. E. & Ziff, E. B. (1984) *Nature (London)* **311**, 433–438.
14. Rappolee, D. A., Mark, D., Banda, M. J. & Werb, Z. (1988) *Science* **241**, 708–712.
15. Rappolee, D. A., Wang, A., Mark, D. & Werb, Z. (1989) *J. Cell. Biochem.* **39**, 1–11.
16. Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159.
17. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J., Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
18. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B. & Erlich, H. A. (1988) *Science* **239**, 487–491.
19. Southern, E. (1975) *J. Mol. Biol.* **98**, 503–517.
20. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
21. Bradshaw, H. D., Jr., & Deininger, P. L. (1984) *Mol. Cell. Biol.* **4**, 2316–2320.
22. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
23. Flemington, E., Bradshaw, H. D., Jr., Traina-Dorge, V., Slagel, V. & Deininger, P. L. (1987) *Gene* **52**, 267–277.
24. Owen, T. A., Cosenza, S. C., Soprano, D. R. & Soprano, K. J. (1987) *J. Biol. Chem.* **262**, 15111–15117.
25. Cosenza, S. C., Owen, T. A., Soprano, D. A. & Soprano, K. J. (1988) *J. Biol. Chem.* **263**, 12751–12758.
26. Augenlicht, L. H. & Baserga, R. (1974) *Exp. Cell Res.* **89**, 255–262.
27. Ferrari, S., Calabretta, B., Battini, R., Cosenza, S. C., Owen, T. A., Soprano, K. J. & Baserga, R. (1987) *Exp. Cell Res.* **174**, 25–33.
28. Miska, D. & Bosmann, H. B. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1140–1145.
29. Rovera, G., Santoli, D. & Damsky, C. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 2779–2783.