occurred between the red and green photopigment genes in the lineage leading to humans and at least twice between color photopigment alleles in NWMs. These repeated divergences and the required parallel amino acid substitutions would indicate adaptive evolution. In addition, the antiquity of the alleles implies that they have been maintained by balancing selection; otherwise, one or two of the alleles would have been lost through random drift (*20*). On the other hand, according to the single origin scenario, the triallelic system should be more ancient than the divergence of the two NWM species, which has been estimated at 16.4 to 19.0 Ma (*13*). Figure 1B suggests that at least seven gaps have been transferred and homogenized among the three alleles in each of the two species. This implies that the critical amino acids that define these alleles have been maintained in the presence of frequent homogenization events, which again suggests balancing selection. The type of selection is probably not minority advantage because the alleles in each species, with the possible exception of the marmoset P556, are maintained at high frequencies (*4*, *5*). One simple explanation is overdominant selection; trichromacy, which occurs in (female) heterozygotes, is thought to facilitate the detection of colored fruits against dappled foliage (*21*). The advantage of having three instead of two polymorphic alleles is an increase in the frequency of heterozygotes and thus the chance of overdominant selection. Another possible advantage of polymorphism is that monkeys with different spectral sensitivities may explore visually different environments (*3*). An additional advantage of the polymorphic system is that dichromats (males and homozygous females) detect color-camouflaged objects better than do trichromats (*22*). Thus, NWMs, which search for fruits cooperatively in groups, enjoy the advantages of both trichromacy and dichromacy (*6*).

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Functional Characterization and Developmental Regulation of Mouse Telomerase RNA

María A. Blasco, Walter Funk, Bryant Villeponteau, Carol W. Greider*

Telomerase synthesizes telomeric DNA repeats onto chromosome ends de novo. The mouse telomerase RNA component was cloned and contained only 65 percent sequence identity with the human telomerase RNA. Alteration of the template region in vivo generated altered telomerase products. The shorter template regions of the mouse and other rodent telomerase RNAs could account for the shorter distribution of products (processivity) generated by the mouse enzyme relative to the human telomerase. Amounts of telomerase RNA increased in immortal cells derived from primary mouse fibroblasts. RNA was detected in all newborn mouse tissues tested but was decreased during postnatal development.

Telomerase is a ribonucleoprotein DNA polymerase that maintains telomere length by adding telomeric sequences onto chromosome ends (*1*). Human and mouse telomerases differ in both their functional properties and their regulation. Partially purified mouse telomerase adds predominantly only one repeat onto a telomeric primer in vitro, whereas the human enzyme adds hundreds of repeats under identical conditions and mixing extracts does not alter the processivity of either enzyme (*2*). In contrast to most normal human tissues, some normal mouse somatic tissues have detectable telomerase activity (*3*). Mouse cells can spontaneously immortalize in culture, whereas human cells rarely, if ever, spontaneously immortalize (*4*). The differential regulation of mouse and human telomerase may affect the ease of immortalization of mouse cells in culture (*3*).

To investigate the difference between the human and mouse enzymes, we cloned the mouse telomerase RNA component. A mouse genomic clone was identified (*5*) by hybridization to a 450-nucleotide (nt) probe from the transcribed region of the human telomerase RNA gene. The transcribed region of the mouse gene was 65% identical to the human telomerase RNA gene (*6*), which indicates that this clone might be the mouse telomerase RNA gene (Fig. 1) (*7*). The sequence identity in the transcribed region of the human and putative mouse RNA genes is significantly less than that

M. A. Blasco and C. W. Greider, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724, USA.
W. Funk and B. Villeponteau, Geron Corporation, Menlo Park, CA 94025, USA.

*To whom correspondence should be addressed.

found between other small RNA genes in humans and mice (*8*).

The potential template regions of the human and mouse RNAs were not completely conserved. Eleven nucleotides can serve as potential templates in the human RNA, whereas only nine nucleotides are possible templates in mice, of which only eight probably serve as templates. Using PCR (polymerase chain reaction) and sequencing, we determined that the rat and Chinese hamster telomerase RNAs contain only eight potential template residues (*9*). The shorter template region in the rodent RNAs may decrease in vitro processivity of the mouse and rat telomerases relative to the human enzyme (*2*). The probability of dissociation of a growing primer from telomerase may be greater for the rodent than for the human telomerase.

Ciliate telomerases are inhibited by, or can use as primers, antisense oligonucleotides that hybridize at or just 3′ of the RNA template (*10*–*12*). We tested the effects of antisense oligonucleotides on mouse telomerase activity (Fig. 2A) (*13*). For the inhibition assays, each oligonucleotide was pre-incubated with mouse telomerase before the primer $d(TTAGGG)_3$ was added (Fig. 2B). The oligonucleotide, MI-2, that covers the template region efficiently inhibited telomerase. To determine the specificity of MI-2 inhibition, we changed the sequence of this oligonucleotide independently at either the 5′ end (generating MI-3) or at the 3′ end (generating MI-5), so that complementarity to the RNA was abolished. These oligonucleotides no longer inhibited telomerase, although MI-3 was itself a substrate for elongation, probably because it ends in a telomeric repeat (Fig. 2C).

Fig. 1. Sequence of the genomic clone encoding the mouse telomerase RNA (mTR) gene. The template region is shown with a box. The estimated positions of the 5′ and 3′ ends of the RNA are designated (*7*). The primers used to map the 3′ end by RT-PCR are shown as arrows above the sequence that they hybridize to. The 3′-most primer that gave a product is shown with a solid line, and two primers that did not give products are shown with dashed lines.

Incubation with MP-1 or two other oligonucleotides, MP-2 and MP-3 (*13*), that hybridized to a region 3′ to the template did not inhibit elongation (Fig. 2B). However, MP-1, whose 3′ end hybridizes just adjacent to the RNA template, was elongated by eight residues (Fig. 2, C and D). When the sequence of MP-1 was lengthened to extend across the template (oligonucleotide MI-4), $d(TTAGGG)_3$ elongation was inhibited (Fig. 2B) and MI-4 did not serve as a substrate for elongation (Fig. 2C). The inhibition and elongation properties of the antisense oligonucleotides, together with other criteria (*14*), are consistent with the cloned RNA being a functional component of mouse telomerase.

To determine which potential template residues are copied, we carried out telomerase reactions with MP-1, substituting dideoxythymidine triphosphate (ddTTP) for deoxythymidine triphosphate (dTTP) (Fig. 2D). The 8-nt labeled product seen with dTTP was reduced to 5 nt with ddTTP, which is consistent with the addition of AGGGTTAG. If the entire potential template region of the mouse RNA, 5′-CCUAACCCU-3′, were copied, 9 nt should have been added to the MP-1 primer. Thus, the 5′-most C in the mouse RNA, which is not conserved in other rodent RNAs, does not serve as a template residue.

Mutations of the telomerase RNA template in *Tetrahymena*, yeast, and humans generate telomerase with altered specificity (*6*, *15*, *16*). We carried out similar template mutagenesis with the mouse RNA gene (*17*). The template region of the genomic clone was changed to specify $(TTGGGG)_n$ repeats instead of $(TTAGGG)_n$ repeats (*18*), and after transfection into NIH 3T3 cells, stable clones were selected. If the mutant RNA altered the specificity of telomerase, cell extracts should contain both a wild-type telomerase that will be inhibited by dideoxyadenosine triphosphate (ddATP) and a mutant telomerase that should be insensitive to ddATP (*6*). Telomerase activity was assayed (*2*) with control and transfected cell extracts with the use of dTTP and deoxyguanosine triphosphate (dGTP) in all reactions and deoxyadenosine triphosphate (dATP) or ddATP in selected reactions (*19*). The products from the telomerase reactions were then amplified by PCR (*20*) in reactions containing all four deoxynucleo-

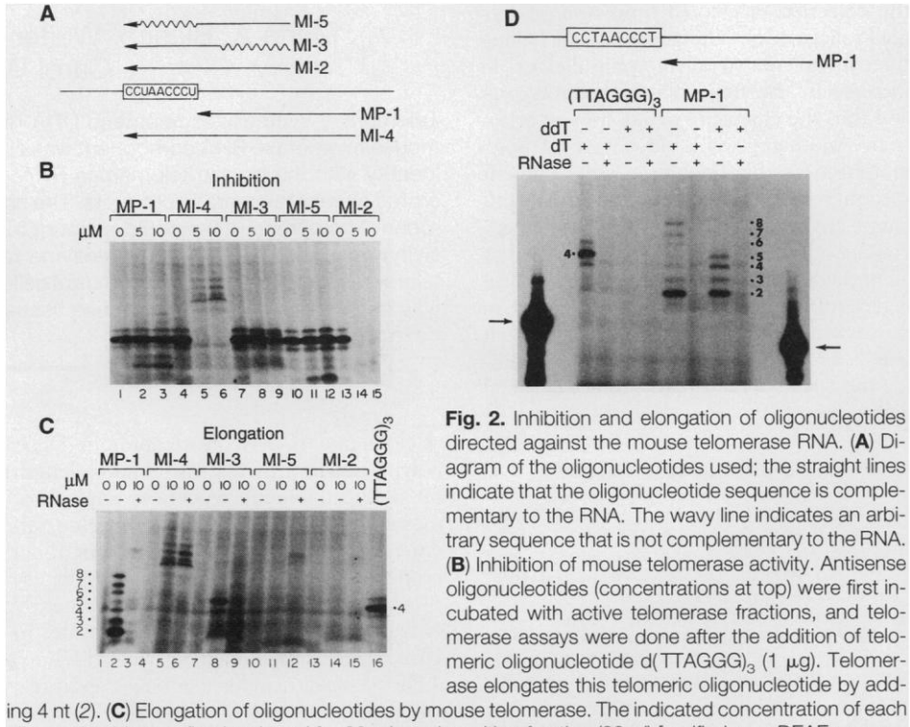

Fig. 2. Inhibition and elongation of oligonucleotides directed against the mouse telomerase RNA. (**A**) Diagram of the oligonucleotides used; the straight lines indicate that the oligonucleotide sequence is complementary to the RNA. The wavy line indicates an arbitrary sequence that is not complementary to the RNA. (**B**) Inhibition of mouse telomerase activity. Antisense oligonucleotides (concentrations at top) were first incubated with active telomerase fractions, and telomerase assays were done after the addition of telomeric oligonucleotide $d(TTAGGG)_3$ (1 μg). Telomerase elongates this telomeric oligonucleotide by adding 4 nt (*2*). (**C**) Elongation of oligonucleotides by mouse telomerase. The indicated concentration of each oligonucleotide was first incubated for 30 min on ice with a fraction (20 μl) [purified on a DEAE-agarose column (*3*)] containing telomerase activity that had been previously treated (+) or not (−) with RNase. After the preincubation, the reaction mix was added and telomerase reactions were carried out as described (*2*). The numbers on the side indicate the size in nucleotides of the elongated products. (**D**) To define the sequence added to the MP-1 oligonucleotide, telomerase assays were carried out with 1 μg of either a telomeric oligonucleotide $d(TTAGGG)_3$ or MP-1 oligonucleotide as primers. Telomerase assays were done as described above except that dTTP (dT) was substituted with ddTTP (ddT) in lanes marked with a "+." Some lanes contain fractions that were first treated with RNase A (+). The number of nucleotides added to each primer is indicated on the sides; arrow on left, $^{32}P(T_2AG_3)$; arrow on right, ^{32}P–MP-1.

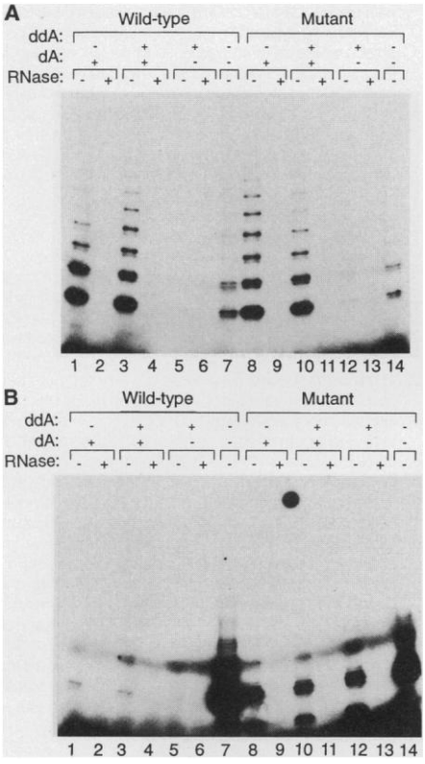

Fig. 3. Synthesis of TTGGGG repeats by mutant telomerase. Extracts from cells containing the wild-type or mutant telomerase clone were assayed with the conventional telomerase assay and the TS primer in the presence or absence of dATP (dA) or ddATP (ddA). Products were amplified by PCR with all four dNTPs present with the use of the TS primer and either a A_2TC_3 primer (**A**) or a A_2C_4 primer (**B**). Each reaction was done with (+) or without (−) a pretreatment with RNase to determine if products were generated by telomerase. The PCR conditions used are described (*19*). In the presence of ddATP in the telomerase reaction, products were not generated by the wild-type enzyme [(A), lanes 5 and 6]; however, products were generated by the mutant telomerase [(B), lanes 10 to 13]. For the wild-type enzyme, addition of both dATP and ddATP did not result in ddATP termination [(A), lanes 3 and 4], and simple omission of dATP did not completely inhibit product synthesis, which suggests that a small amount of dATP may be present in the extracts used.

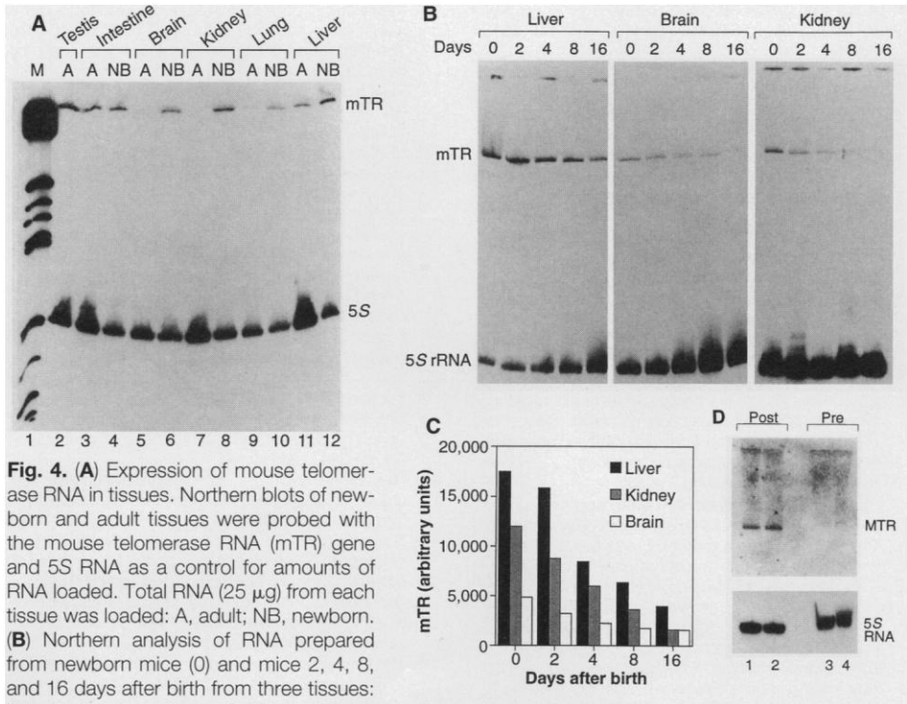

Fig. 4. (**A**) Expression of mouse telomerase RNA in tissues. Northern blots of newborn and adult tissues were probed with the mouse telomerase RNA (mTR) gene and 5*S* RNA as a control for amounts of RNA loaded. Total RNA (25 μg) from each tissue was loaded: A, adult; NB, newborn. (**B**) Northern analysis of RNA prepared from newborn mice (0) and mice 2, 4, 8, and 16 days after birth from three tissues: liver, brain, and kidney; 5*S* rRNA, 5*S* ribosomal RNA. (**C**) Comparison of amounts of telomerase RNA in different tissues. The data shown in (B) were analyzed on a BAS2000 phosphorimager (Fuji Bio-Imaging) and the mTR signal was normalized to the 5*S* RNA signal. The relative amount of RNA is expressed in arbitrary units. (**D**) Mouse telomerase RNA expression in primary and immortal *Mus spretus* fibroblasts. Total RNA (20 μg) was isolated either before crisis (Pre) or after crisis (Post) from *Mus spretus* fibroblasts (*2*) and was loaded in duplicate lanes and separated by polyacrylamide gel electrophoresis. For all experiments, the RNA was separated on a 6% polyacrylamide, 7 M urea gel, and the RNA was electroblotted to Hybond membrane (Amersham) and probed with the mouse telomerase RNA gene and the 5*S* RNA gene.

side triphosphates (dNTPs) and a return primer specific for either wild-type repeats [$(A_2TC_3)_3$; Fig. 3A] or for mutant repeats [$(A_2C_4)_3$; Fig. 3B]. As expected, the wild-type primer amplified telomerase products in both control and transfected cells (Fig. 3A, lanes 1 and 8), and products were not seen in reactions in which dATP was substituted by ddATP (Fig. 3A, lanes 5 and 12). In contrast, when similar reactions were performed with the mutant PCR primer, only the cells containing the mutant RNA had activity in the presence of ddATP (Fig. 3B, lane 12). Thus, a mutation in the cloned RNA template region generated the expected mutant telomerase products, providing strong evidence that this RNA is the mouse telomerase RNA component (mTR).

To study the regulation of the mouse telomerase, we followed mTR expression in tissues and cell lines. Northern (RNA) blots of total RNA from various tissues from both newborn and adult mice were probed with mTR and with a 5*S* RNA gene to allow normalization to total RNA (Fig. 4A). In newborn mice, mTR was detectable in intestine, brain, kidney, lung, and liver. However, in adult mice, mTR was detected only in testis, intestine, liver, and spleen. Unlike many human somatic tissues that do not express detectable telomerase activity (*20*), many adult mouse tissues including testis, liver, spleen, and kidney contain telomerase activity (*3, 21*). The detection of mTR in some adult tissues correlates with the presence of telomerase activity (*22*).

To identify when during postnatal development amounts of mTR changed, we prepared RNA from three tissues at different postnatal developmental stages up to 16 days after birth (Fig. 4B). The mTR amounts decreased in the brain (68%), kidney (87%), and liver (77%) (Fig. 4C). However, the mTR amounts in the liver were initially higher than those found in the other tissues, and after day 16 the amount of mTR in the liver was equivalent to that in the newborn brain (Fig. 4C). This larger amount of mTR in adult liver is consistent with the presence of telomerase activity in adult mouse liver.

Primary *Mus spretus* fibroblasts lack detectable telomerase activity and show telomere shortening. After immortalization, telomerase activity is detected and telomere length is maintained (*3, 23*). We used these *Mus spretus* cell lines to follow mTR amounts before and after immortalization. Northern blot analysis showed that the immortalized fibroblasts expressed at least 18 times more mTR than the parental fibroblasts, which indicates that the increase in the amount of mTR correlated with the increase in telomerase activity after immortalization (Fig. 4D). The identification of the human and mouse telomerase RNAs will allow a detailed investigation of the mechanism and regulation of mammalian telomerases.

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7. The 5′ end of the mouse RNA was mapped by primer extension and ribonuclease (RNase) protection with the use of standard methods, and the 3′ end was mapped by reverse transcriptase–PCR (RT-PCR). Two primers that hybridize at nt 501 to 518 and nt 523 and 543 did not generate products by RT-PCR; however, the primer that hybridized between nt 413 and 435 did generate RT-PCR products. On the basis of the alignment of the conserved region in the human and mouse RNAs, we estimate that the 3′ end is near position 453 (marked with 3′ in Fig. 1) and that the mouse RNA is ~430 nt long. The GenBank accession number for the mTR sequence is U33831.
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14. To further characterize the function of the cloned mouse sequence, we used several approaches. Genomic Southern (DNA) blots probed at high stringency with the potential transcribed region of the mouse gene identified a single band, which suggests that the gene for the mouse telomerase RNA is a single copy like the human gene (*6*). To confirm that the gene we cloned is expressed, we used RT-PCR from total mouse RNA with or without the initial reverse transcription step. One band with the expected size and sequence was amplified from total RNA after reverse transcription; no band was generated in the absence of reverse transcriptase. The conditions for RT-PCR were as follows. First-strand complementary DNA (cDNA) synthesis was primed by random hexamers, $p(dN_6)$, and the products were amplified by PCR with the use of two specific primers: mTR5b, 5′-CGTCGACTAGGGTCGAGGGCGGCTAGGCCT-3′; and mTR3, 5′-GGAGGCGGCCGCAGGTGCACTTCCCACAGCTCAG-3′. This reaction was followed by a second round of PCR with primers mTR5b and an internal nested primer called "nest B" (5′-GGAGGCGGCCGCAGACGTTTGTTTTTTTGAGGC-3′). Total RNA (3 μg) was mixed with random hexamers (8 ng) in a final volume of 10 μl, denatured at 95°C for 10 min, and chilled on ice and then incubated for 60 min at 50°C. We stopped the reactions by heating the tubes at 95°C for 10 min. The first-strand cDNA product (1 μl) was used for each PCR reaction. PCR reactions contained 1× PCR buffer, 5 mM dNTPs, 100 ng of each primer, 0.1 μg of T4 gene 32 protein, and 2 units of Taq polymerase from Perkin-Elmer. The conditions of PCR amplification were as follows: 94°C for 1 min; 60°C for 45 s; and 72°C for 1.5 min. Typically, 35 to 40 cycles were carried out for a first amplification. For nested amplification, 1 μl of the first PCR product was used in a second PCR. For cloning, the PCR products were phenol-extracted, precipitated, and digested with restriction enzymes Not I and Sal I and cloned in KS+ Bluescript that was previously digested with Not I and Sal I. We also assayed for copurification of the RNA with telomerase activity. We purified mouse telomerase activity over four columns and did Northern blots of RNA extracted from the fractions. An RNA of the expected size copurified with telomerase activity over each column.
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17. Transient transfection of plasmids containing 3.2 kbp of genomic flanking sequence and a 100-bp internal deletion of the transcribed region into NIH 3T3 cells generated an RNA of the expected size on Northern blots, which indicates that the cloned genomic DNA is sufficient to direct transcription of mTR.
18. The mutant mouse telomerase RNA gene with the template sequence 5′-CCCAACCCCAA-3′ was generated by in vitro mutagenesis (Amersham) and cloned into Bluescript in the context of a 5.5-kb genomic mTR clone. The mTR gene coding region was sequenced to confirm the template mutation.
19. For both wild-type and mutant extracts, a conventional telomerase reaction was done as described (*2*) with the use of the TS primer (*20*), dGTP (2 mM), dTTP (2 mM), and for some reactions dATP (2 mM) or ddATP (0.5 mM) as indicated (Fig. 3). Telomerase products were then amplified with the TS primer and either a $(A_2TC_3)_3$ primer or a $(A_2C_4)_3$ primer. Hot star PCR was used with the following conditions: 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min for 25 cycles.
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22. Although mTR was not detected in the adult mouse kidney in this study, a low level of telomerase activity in this tissue has been reported (*3*). Such a difference may be attributed to the low activity levels detected or to the low sensitivity of Northern blot analysis of mTR.
23. The mouse species *Mus spretus* was used here because the standard laboratory species *Mus musculus* has very long telomeres that make detection of telomere length changes difficult [D. Kipling and H. J. Cooke, *Nature* **347**, 400 (1990)].
24. We thank S. K. Smith and R. Adams for excellent technical assistance, J. Rodriguez and A. Samuelson for generating PCR fragments from rat and hamster telomerase RNAs, J. Feng and W. Andrews for providing the original TRC3 clone; A. Zaug and T. Cech for advice about the 3′ end; D. Hanahan for critical reading of the manuscript, K. Collins and M. Serrano for helpful discussions; and R. DePinho for providing RNA . This work was supported by NIH grant AG09383 (C.W.G.), the Allied Signal Award for Aging Research, Geron Corporation, and the Ministerio de Educación y Ciencia from Spain (M.A.B.).

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An Essential Role for Rho, Rac, and Cdc42 GTPases in Cell Cycle Progression Through G_1

Michael F. Olson, Alan Ashworth, Alan Hall

Members of the Rho family of small guanosine triphosphatases (GTPases) regulate the organization of the actin cytoskeleton; Rho controls the assembly of actin stress fibers and focal adhesion complexes, Rac regulates actin filament accumulation at the plasma membrane to produce lamellipodia and membrane ruffles, and Cdc42 stimulates the formation of filopodia. When microinjected into quiescent fibroblasts, Rho, Rac, and Cdc42 stimulated cell cycle progression through G_1 and subsequent DNA synthesis. Furthermore, microinjection of dominant negative forms of Rac and Cdc42 or of the Rho inhibitor C3 transferase blocked serum-induced DNA synthesis. Unlike Ras, none of the Rho GTPases activated the mitogen-activated protein kinase (MAPK) cascade that contains the protein kinases c-Raf1, MEK (MAPK or ERK kinase), and ERK (extracellular signal–regulated kinase). Instead, Rac and Cdc42, but not Rho, stimulated a distinct MAP kinase, the c-Jun kinase JNK/SAPK (Jun NH_2-terminal kinase or stress-activated protein kinase). Rho, Rac, and Cdc42 control signal transduction pathways that are essential for cell growth.

Constitutively active V12Cdc42 (Cdc42 with valine substituted for glycine at position 12), V14Rho, V12Rac, and V12Ras recombinant proteins were each microinjected with rat immunoglobulin G (IgG) into the cytoplasm of quiescent Swiss 3T3 fibroblasts, and the incorporation of bromodeoxyuridine (BrdU) into nascent DNA was measured after 40 to 48 hours (*1*). Microinjection of rat IgG alone (Fig. 1, A and B) had no effect on DNA synthesis; ~2% of the cells showed BrdU incorporation (Fig. 1C). Microinjection of V12Cdc42 efficiently stimulated DNA synthesis (Fig. 1, A and B); ~90% of injected cells were positive for BrdU incorporation (Fig. 1C). V12Ras, V14Rho, and V12Rac also stimulated BrdU incorporation in the majority of the injected cells (Fig. 1C).

M. F. Olson and A. Hall, CRC Signal Transduction and Oncogene Group, Medical Research Council Laboratory for Molecular Cell Biology and Department of Biochemistry, University College London, London WC1E 6BT, UK. A. Ashworth, Chester Beatty Laboratories, Institute of Cancer Research, Fulham Road, London SW3 6JB, UK.