Expression of Replication Factor C 40-kDa Subunit Is Down-Regulated During Neonatal Development In Rat Ventricular Myocardium

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Abstract During neonatal development, cardiac myocytes undergo a transition from hyperplastic to hypertrophic growth. Whether these cells are terminally differentiated and permanently withdrawn from the cell cycle shortly after birth is controversial. Nevertheless, the clinical observation that functionally significant myocardial regeneration has not been documented in cardiovascular disease or injury during adulthood seems to support the notion that the vast majority of cardiac myocytes do not proliferate once they differentiate. Regardless of the controversy, the elucidation on how mitosis is blocked in cardiac myocytes may facilitate development of new cardiovascular therapies, based on the regeneration of the adult myocardium. To better understand postnatal myocardial development, we performed suppression subtractive hybridization to isolate genes that are differentially expressed in day one or day seven postnatal rat ventricular myocardium. Here we report the down-regulated mRNA expression of the 40-kDa subunit of replication factor C (RFC p40 or RFC2), which is an essential processive factor for proliferating cellular nuclear antigen-dependent DNA replication during neonatal myocardial development. J. Cell. Biochem. 78:533–540, 2000. © 2000 Wiley-Liss, Inc.

Key words: terminal differentiation; heart development; replication factor C; suppression subtractive hybridization

During neonatal development, cardiac myocytes in man, mouse, and rat undergo a transition from hyperplastic to hypertrophic growth, such that further increases in myocardial mass are typically not accompanied by cardiac myocyte proliferation [Soonpaa and Field, 1998]. Therefore, they respond to growth, increased workload, and injury mainly by hypertrophy. The concept that cardiac myocytes are terminally differentiated and cannot proliferate once cell division ceases shortly after birth in the mammalian heart is still under debate [Anversa and Kajstura, 1998]. There are evidences demonstrating that cardiac myocytes maintain low but measurable levels of DNA replication

Received 20 January 2000; Accepted 14 March 2000

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beyond the perinatal period in humans [Quaini et al., 1994] and rats [Overy and Priest, 1966; Cheng et al., 1995]. Nevertheless, the clinical observation that functionally significant myocardial regeneration has not been documented in cardiovascular diseases and injuries that result in cardiac myocyte loss seems to support the notion that the vast majority of cardiac myocytes do not proliferate once they differentiate. Unlike adult skeletal muscle that has satellite cells, which can be stimulated to proliferate and develop into mature myocytes, mammalian adult cardiac muscle lacks progenitors and thus cannot regenerate. Consequently, myocardial loss due to injury or disease during adulthood is irreversible.

Currently, the exact mechanism that underlies the transition from hyperplastic to hypertrophic growth of cardiac myocytes in mammalian heart remains largely unknown [Olson and Srivastava, 1996; Anversa and Kajstura, 1998]. Regardless of the controversy whether cardiac myocytes undergo terminal differentiation, the elucidation on how mitosis is blocked in cardiac myocytes may facilitate development of new therapies for cardiovascular injury or

Sequence data have been deposited with the GenBank/ EMBL Data Libraries under the accession number AF208499.

Grant sponsor: Research Grants Council of the Hong Kong Special Administrative Region; Grant number: CUHK4305/99M.

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diseases, based on the regeneration of the adult myocardium.

Previous studies suggested that most cardiac myocytes in rat heart gradually cease to undergo DNA replication, which is a prerequisite for proliferation, within the first two weeks after birth [Clubb and Bishop, 1984; Li et al., 1996]. Myocardial growth from this time is primarily due to hypertrophy of existing myocytes [Engelmann and Gerrity, 1988]. In contrast to the relatively well-characterized process that controls myocyte hypertrophy, investigations that have focused on determining the mechanisms regulating the loss of myocyte proliferation during postnatal myocardial development are limited. Recent studies have shown that the activities of cyclin-dependent kinases (CDKs) 2, 4, and 6 were down-regulated in day two postnatal cardiac myocytes [Flink et al., 1998]. Interestingly, results of another report showed that serum stimulation promoted the G1 CDK activities without induction of DNA synthesis in rat neonatal cardiac myocytes [Tamamori et al., 1998]. In skeletal myoblasts, differentiation is associated with induction of p21, a CDK inhibitor, by a MyoD-dependent pathway. Although p21 has been found to be induced in rat neonatal cardiac myocytes [Flink et al., 1998], cardiac muscle has not been shown to express any myogenic determining basic helix loop helix factors like MyoD or any proteins that are functionally similar [Skerjanc and McBurney, 1994]. Moreover, growth factor receptors in differentiated cardiac myocytes appear to be intact with up-regulation of immediate early response genes, like c-myc, with pressure overload-induced cardiac hypertrophy [Izumo et al., 1988]. This implies that critical factors for myocardial differentiation are probably signal-transduction proteins or proteins related to gene transcription and DNA replication. We have initiated a project to characterize gene expression in various developmental states of the cardiovascular system by highthroughput sequencing of randomly selected clones from human heart cDNA libraries to generate expressed sequence tags [Liew et al., 1994; Hwang et al., 1995; Hwang et al., 1997]. Many developmentally regulated genes in the human heart have been isolated and characterized [Tsui et al., 1994; Chan et al., 1998; Luk et al., 1998; Kotaka et al., 1999]. To better understand the loss of cardiac myocyte proliferation during postnatal myocardial development, we

recently embarked on the isolation of genes that were differentially expressed in day one and day seven postnatal Sprague-Dawley rat ventricular myocardium, employing the technique of suppression subtractive hybridization (SSH) [Diatchenko et al., 1996; Diatchenko et al., 1999]. In this article, we report the downregulated expression of a mRNA that encodes the rat homologue of human replication factor C 40-kDa subunit (RFC p40 or RFC2) during neonatal development of the rat ventricular myocardium, as identified by the method of SSH.

MATERIALS AND METHODS

Experimental Animals and Sampling

Sprague-Dawley rats of different ages were obtained from the animal facilities of our university. Hearts were carefully dissected from decapitated animals and rinsed in phosphate buffered saline. Only the lower two-thirds of the myocardium, which contains mostly ventricular cells, was used for RNA isolation.

Total RNA and Polyadenylated RNA Isolation

Total RNA was extracted from rat ventricular myocardium of different ages using TRIZOL reagents (Life Technologies, Gaithersburg, MD), and then polyadenylated RNA was isolated using PolyATtract mRNA Isolation System (Promega, Madison, WI), according to manufacturers' specifications. Quantity and quality of each sample were determined spectrophotometrically by A260 and A260/280 ratio, and checked by electrophoresis on a 1.2% agarose/formaldehyde gel.

SSH, Forward and Reverse Subtraction

Suppression subtractive hybridization was performed using polymerase chain reaction (PCR)-Select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to manufacturer's protocol. We used day one rat ventricular myocardium cDNA as tester and day seven cDNA as driver for forward subtraction to identify day one-dominating cDNA, and vice versa for reverse subtraction to identify day sevendominating cDNA. Unsubtracted control for each subtraction was prepared by the same procedures but without the subtractive hybridization step.

Construction of Subtracted Library, Differential Screening, and Dot-Blot Analysis

Forward- and reverse-subtracted cDNAs were TA-cloned into Clontech's pT-Adv vectors and transformed into the Escherichia coli strain DH5α to generate two subtracted libraries, each with about 1,500 colonies harboring inserts encoding for putative candidate cDNA fragments that dominate in either day one or day seven postnatal rat myocardium. To eliminate a majority of the false positives, the libraries were prescreened by colony hybridization using forward-subtracted and reversesubtracted probes, which were RsaI-restricted subtractive-hybridized cDNA. Briefly, 753 randomly selected colonies were double-spotted identically on two 8 cm imes 12 cm Immobilon-N PVDF membranes (Millipore, Bedford, MA), and hybridized at 72°C overnight with either the $[\alpha^{-32}P]$ dCTP random-primed labeled forward- or reverse-subtracted probe in ExpressHyb Hybridization Solution (Clontech, Palo Alto, CA), then washed several times until a final stringency of 68° C in $0.2 \times$ SSC, 0.5%sodium dodecyl sulfate (SDS) was achieved. These membranes were then exposed to autoradiography film at -80° C with intensifying screens for four days to two weeks. Only the forward-subtracted clones that hybridized substantially stronger with the forwardsubtracted probe than the reverse-subtracted probe were further investigated, and vice versa for the reverse-subtracted candidates. The cDNA inserts of more than 250 putative differentially expressed clones were amplified by PCR, arrayed on Immobilon-N membrane and further screened by dot-blot analysis, using cDNA probe incorporated with $[\alpha^{-32}P]$ dCTP and $[\alpha^{-32}P]$ dATP during the first strand synthesis either from day one or day seven RNA from rat myocardium. The same hybridization and washing procedures described above were also applied for these dot-blots. The intensity volumes of hybridization signals were measured by densitometer (Molecular Dynamics, Sunnyvale, CA).

Northern Blot Analysis

Total RNA of approximately 20 μ g from rat ventricular myocardium of different ages were electrophoresed on a 1.2% agarose/formaldehyde gel, and blotted in 10× SSC onto Immobilon-N PVDF membranes by overnight capillary action. The cDNA probes were derived from the PCR products of B-actin (forward: 5'-TCACCAACTGGGATGACATG-3'; reverse: 5'-GATGTCCACGTCACACTTCAT-3'), RFC p140 (forward: 5'-CACTTCAGGTGGTA-AAGGCATCGA-3'; reverse: 5'-TAGGGCATC-CTGCTCTTTCTCATC-3'), and RFC p40. After gel purification, cDNA probes were randomprimed labeled with $[\alpha^{-32}P]$ dCTP using Ready-To-Go DNA labeling beads (Amersham Pharmacia Biotech., Buckinghamshire, England). The blots were hybridized at 65°C in ExpressHyb Hybridization Solution and washed several times until a final stringency of 50°C in $0.1 \times$ SSC, 0.1% SDS was achieved. The blots were then exposed to autoradiography film at -80°C with intensifying screens for one to four days. Normalization was achieved by hybridization of β -actin cDNA probe to the same membrane after stripping. The band intensities were quantified by densitometer.

RESULTS

Among the 753 clones randomly picked for prescreening by colony hybridization, 142 forward-subtracted clones, which hybridized substantially stronger with the forwardsubtracted probe than with the reversesubtracted probe, and 155 reverse-subtracted clones, which hybridized substantially stronger with the reverse-subtracted probe than with the forward-subtracted probe, were selected as the putative differentially expressed clones (Fig. 1A). Dot-blot analysis further revealed that the day one cDNA probe hybridized to one of the forward-subtracted clones with a 2.75-fold higher intensity relative to the day seven cDNA (Fig. 1B). Northern blot analysis showed a 2.33-fold down-regulation of a 2.0-kb transcript encoded by this forward-subtracted cDNA clone during day one and day seven postnatal in the rat ventricular myocardium (Fig. 2). This cDNA clone, which is 0.8 kb in size, is 87% identical to the nucleotide sequence of replication factor C (activator 1) 40-kDa subunit (RFC p40 or RFC2) of human (GenBank/EMBL accession number M87338), and 94% to that of mouse (GenBank/EMBL accession number AF139987) (Fig. 3A). When the partial coding sequence of our cDNA clone was translated and aligned with the amino acid sequences of human RFC p40 and mouse RFC p40, we found that it encodes 216 amino acids whose sequence is 95% and 98% identical to the corre-



Fig. 1. A: Colony hybridization of subtracted clones (16.7% shown) with forward-subtracted (left panel) and reversesubtracted (right panel) probes. Each clone was double-spotted on two identical membranes. In each 4×4 matrix, rows 1 and 3 were double-spotted with four forward-subtracted clones. Rows 2 and 4 mostly contained reverse-subtracted clones, but were embedded with forward-subtracted clones on A1, B1, C1, D1, A2 and B2 of the right hand portion of row 4. Like many other forward-subtracted clones, RFC p40 (arrow) hybridized substantially stronger with the forward-subtracted than the reverse-subtracted probe. B: Dot-blot analysis of RFC p40 showed a 2.75-fold stronger signal when hybridized with the day one rat ventricular myocardium cDNA probe (left), compared to the day seven cDNA probe (right). Signal intensities were quantified by densitometer and indicated below the dots. After normalization with the β -actin signals of the same day, a 2.75-fold reduction in RFC p40 signal was observed.

sponding C-terminus of the human and mouse RFC p40 proteins, respectively (Fig. 3B). So we conclude that this forward-subtracted cDNA, which is down-regulated in day seven relative to day one postnatal by 2.33-fold on Northern blot analysis, partially encodes the rat homologue of RFC p40, which has not been reported before.

Because many of the catalytic functions of the heteropentameric RFC complex reside in the large subunit (RFC p140), we decided to investigate if there is any down-regulation of this transcript besides the RFC p40 mRNA. As revealed by Northern blot analysis, the level of RFC p140 mRNA, whose transcript was about 5.0 kb in size, though fluctuated during the course and decreased by about 30% on day five, was just about the same on day one, day three, and day seven (Fig. 2B). In contrast to RFC p140, RFC p40 dropped smoothly during the course and decreased by more than two-fold on day seven compared to day one.

DISCUSSION

In spite of the controversy whether cardiac myocytes are terminally differentiated, it is believed that myocardial differentiation involves withdrawal from the cell cycle. Adult mammalian cardiac myocytes were thought to be incapable of proliferation because the vast majority of them gradually cease to undergo DNA replication, which is essential for proliferation, during neonatal development. In rat, this occurs within the first two weeks after birth [Clubb and Bishop, 1984; Li et al., 1996]. As suggested by their growth pattern in terms of cell volume, number, and the degree of binucleation, cardiac myocytes undergo a rapid transition from hyperplastic to hypertrophic growth around day three to day four postnatal (Li et al., 1996). Employing the technique of SSH, we reported here the down-regulated expression of a mRNA, which encodes the rat homologue of the 40-kDa subunit of replication factor C (RFC p40 or RFC2), in rat ventricular myocardium on day seven compared to day one postnatal. This down-regulation is concurrent with the course of myocardial differentiation.

Replication factor C is essential for processive DNA replication in eukaryotes [Tsurimoto and Stillman, 1989]. The RFC complex in human consists of five distinct subunits, namely p140, p40, p38, p37, and p36, designated according to their migration in SDS/PAGE, in accordance with the common nomenclature in literature [Chen et al., 1992a; Chen et al., 1992b; Bunz et al. 1993; O'Donnell et al., 1993]. Although human and mouse RFC p40 is sometimes referred as RFC2 (GenBank/EMBL accession numbers NM_002914, M87338, AF139987), RFC p40 is homologous to RFC4 in Saccharomyces cerevisiae, which adopts a slightly different nomenclature [Cullman et al., 1995].

Replication factor C serves as an ATPdependent clamp loader for loading the homotrimer sliding clamp, proliferating cellular nuclear antigen (PCNA), onto DNA during DNA replication. RFC first binds specifically to a DNA primer end and then recruits PCNA and loads it onto DNA in an ATP-dependent manner [Lee et al., 1991; Tsurimoto and Stillmann, 1991]. Subsequent ATP hydrolysis is



Fig. 2. A: Northern blot analysis of RFC p140, RFC p40, and β -actin on day one (lane 1), day three (lane 2), day five (lane 3), and day seven (lane 4) rat ventricular myocardium RNA. **B:** The band intensities were quantified by densitometer and expressed as arbitrary units relative to the β -actin signal of the same day.

necessary for the polymerase to enter the complex and for initiation of chain elongation [Lee and Hurwitz, 1990; Burgers, 1991].

In vitro reconstitution of human RFC demonstrated that all five subunits were required to form a stable, active complex for PCNAdependent DNA synthesis [Uhlmann et al., 1996]. Despite the striking sequence redundancy among the four small subunits, deletion of the C-terminal part from them abolished their ability to form the RFC complex [Uhlmann et al., 1997], explaining why all five subunits are required. Assembly of RFC was observed to involve distinct subunit interaction in which p36 interacted with p37 and p40 interacted with the p37·p36 subcomplex [Ellison and Stillman, 1998]. This subsequent core subcomplex of p40·p37·p36 was stable enough to be isolated and purified, and contained DNAdependent ATPase activity that was stimulated by PCNA, although this three-subunit core complex did not load PCNA onto DNA, in contrast to the five-subunit RFC [Cai et al., 1997]. A mutation in any one of the ATP binding sites of the p36, p37, p40, or p140 subunits markedly reduced replication activity of the RFC complex and the ATPase activity of the RFC or the core p40·p37·p36 subcomplex [Cai et al., 1998]. The core p40·p37·p36 complex then binds the p38 subunit, which is essential for the interaction between this core complex and the large p140 subunit [Uhlmann et al., 1996].

Among the subunits, RFC p40 appears to play an important role in ATP hydrolysis. Among the p40, p37, and p36 subunits, only p40 possessed weak ATPase activity, and only subcomplexes containing p40 were active as an ATPase [Podust et al., 1998]. In the light of these previous findings, and that the RFC complex most likely contains equimolar levels of each subunit [Uhlmann et al., 1996], the downregulation of RFC p40 during the course of myocardial differentiation would imply a decrease in the PCNA-dependent DNA replication, consistent with the notion that the vast majority of cardiac myocytes seldom replicate DNA shortly after birth. Though it has been known previously by virtues of mitotic figures, thymidine incorporation, BrdU and PCNA immunochemistry, that differentiated cardiac myocytes scarcely undergo DNA replication, we report here that the mRNA level of an essential processive factor for DNA replication decreases gradually during neonatal development in rat heart.

Surprisingly, in contrast to the downregulated mRNA expression to p40, the large

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Human Mouse Rat	CANTTGAGTTTCCATTTCTCGGATTGGGAACTGGTATAAGCATTGTCTGTGATGTAAA	60 0 0
Human Mouse Rat	CAAAGTCTTCAATATTTGGAGAAAACATCTCCTCATACTTGAGAGCACAAGAAGAAGAAGAAG	120 0 0
Human Mouse Rat	GAGACCCTCACTGCTGGGGAGTCCCTGCCACACCACACTGAATCGGAA	180 0 0
Human Mouse Rat	START TTCCGAGACBGAGAGAGGACCCCCAGAATGGAGGCCACCTCTCGTCGTCCCCCGCC 	240 51 0
Human Mouse Rat	CAGETGEAGEGCEAGEACTCTGACCOTGEGCTTECGTTCAGEAGEGCCUGGGACCGC GA9AGTGEGGCCAG	300 99 0
Human Mouse Rat	GCCCACTACGARCTGCCGTGGGTTGAAAAATATAGGCCACTAAAGCTGAATGAA	360 156 0
Human Mouse Rat	CCCARTGARGACACCCTGACCAGGOTAGAGGECTTTGCAACGAAGGAAATGTGCCCAAC CCCARTGAAGACACCCTGAGCAGGCTC GAGGTCTGAGACACCCCCCAACGCAATGTGCCCAAT	420 216 0
Human Mouse Rat	NTOATCATTGCCGCCCCTCCAGGAACCGCCAAGACCACAAGAATCTCTCGCCTCGCCCGG ATCATCATTGCTGCGCCCAGGAACGGCAAGACAAGCAGGATCTCTCGCCTGCG 	480 276 0
Human Mouse Rat	CCOTCCTCGGCCCACGACTCAAACEAACCATCTTCGCAACTCAATGCTTCAAATGACAAG CCCTTCCTCGGCCCCGCCCTCAACGACGCCGCACGCCTCGAATGCCTCAAATGACAAG	540 336 0
Human Mouse Rat	econtercetrescartabatteratics concabargecaeterce cconcenter etchegartabattertecc cconcenter etchegartabattertecc	600 396 0
Human Mouse Rat	ANACCCCCACAMAAGATCATCTTCTGGATGAAGCAGACAGCATGACCGACGGAAGCCAG AACGGCCGCCACAAGATCATCATACATGAAGCAGACAGCATGACGGACG	660 456 39
Human Mouse Rat	CAAGCCTCCAGGAGAAACCATCGAAAACCAGCCAGCCCCGTGGGTGG	720 516 99
Human Mouse Rat	AATGCTTCGGATAACATCATGAGCCCATTCAGTCCGCTCTGCAGTCGTCCGCTACAG AATGCTTCAGAGAAAATCATAGAGCCCATCCAGTCCGGCTGTGCGGCTGCTCCGCTACAG AATGCTTCAGACAAAATCATAGAGCCCATCCAGTCCGGCTGTGCGCGGCCGGC	780 576 159
Human Mouse Rat	RACTCACCACCCACHTCCTCACCAGGCTCATGAATGTTATCGACAAAAGA AAGTCACHGACGCCAGGTCCTCACCAGGCTCATGAATGTCAAGAAAGA	840 636 219
Human Mouse Rat	CC <mark>TACACTGATGACGGCCTAGAAGCCATCATCTTCAC</mark> GCCCAGGGAGACATGAGCCAG CC <mark>ATACACAGATGACGGCCTGGAAGCCATAATC</mark> TTCAC <mark>AGCCCAGGAGACATGC</mark> CCAG CC <mark>ATACACAGATGACGCCCTCGAAGCCATCATT</mark> TTCAC <mark>AGCCCAGGGAGACATGCGC</mark> CAG	900 696 279
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Human Mouse Rat	NATGCCNACATTGACGNACCTACAAGATTCTTGCTCACTTGTGGGATGTGGGCTACTCA GACGCCAACATTGACGICGCCTACAAGATTCTTGCTCACCTCTGGGCACCTGGGCTACTCA GACGCCAACATTGATGACGCCTACTACAAGATTCTTGCTCACCTGTGGGACCCTGGGCTACTACA	1080 876 459
Human Mouse Rat	CCAGAAGATATCATTEGCAACATCITTCEACTCIGTAAAACTITCCGAAATGGCAGAATAC CCAGAAGATCICATCGCCATCATTITCCCACGCIGCAAAACCITCCCCATGGCIGAATAC CCAGAAGATCICATCGGCAATATTITCCCGACICIGAAAACCITCCCAATGGCIGAATAC	1140 936 519
Human Mouse Rat	ctganactggactt <mark>a</mark> atcaagbaattggatacactgacatgaaa <mark>ata</mark> scggaaggagtg ttgaaactggactt <mark>c</mark> atcaagbcattggatacactcactgaaac <mark>ac</mark> ggaaggagtg ttgaaactggactt <mark>c</mark> atcaagba <u>c</u> attggatacacccactgaaacgagtggaggaggaggaggaggaggaggaggaggaggagga	1200 996 579
Human Mouse Rat	AACTORUTTTGCAGATGGCASGCTCCTGCGAAGGCTGTGTCAGAAGACAATGGCCCCG AACTOCTGCTGCAGATGGCTGCGCTCCTGGGCAGGCTGTGTCAGAAGACCATGGCTCCA AACTOCTGCTGCGAGATGGCGGCGCTCCTGGGCAGGCTGTGTCAGAAGACGATGGGTCCG GTCR	1260 1056 639
Human Mouse Rat	eteccaretalagacagaagacticactgactgacttacagagaccccattctagagaac eteccaretagagacgeccicactgatgaactagaactagagactttgttetogga etegcorecagaacttgagacgecctcactgatggaactagagaacccttgttetogga	1320 1116 699
Human Mouse Rat	GGAGCCGCGCGTTTCTGATGGGGGAAAATGCCCCTGAGGCGAGCCAACATGACTGTCCC GATGTGCCCCAGCTAGAACATGCTCACTTGTTTTTTTTTT	1380 1176 759
Human Mouse Rat	CANACTCCAGTGGCTGGCCAGGCGCGGTAGTCACGCCTGTATCCCTACACTTGGGAG CCCAGGCTGACCTTGAACTCCCGATCGCCCGAGTGCTGGGGTTGGACGTGTGTGT	1440 1236 819
Human Mouse Rat	CCCGAGGCAGGTCANACACCTGAGGTCAGAAGTTCAAGACCAGCCTGGCCAACATGGGGA CTGTGCTTTCTGCGTGTACTACAGATGGAACCCAGGACTCGTGCGGTGTCAGACGAGCACTG CACCTCCCTCCCAGCTCTGCCTGCCGAGTACTAGGGTCACAGGTGTGTGT	1500 1296 879
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Human Mouse Rat	STTECRETERGECEARGATERACKCATTECACTCCEGCCGACAGAGACTCEARCEG CGATAAAGCCTTTGGATGTT	1680 1436 1059
Human Mouse Rat	<mark>В</mark> GGAAAAAA <mark>B</mark> AAATAAAC <mark>B</mark> CCCG Втаgctgat <u>B</u> AAgCC <mark>B</mark> TTggatgt <u>B</u>	1709 1436 1084

	II	
Ruman	NEVEAVCGGAGEVEAODSDPAPAFSKAPGSAGHYEL	60
Mouse	MEVQESGCDPSESGAQEPSPVPSKTAGHYEL 200V MKYRPLKLNE VGNEDTVSRL	55
Rat		0
Drosophila	MPEEPEKTADDKRSHI PWI DKWRPVKFKENVGNEDTVARL	40
C.elegans	MSKSEKQQIAEWW9KYRPKVLADEVGNENIVER	34
S.cerevisiae	SKTLSLQUWVSKYREOVLSDIVERKETIDR	33
	111 17	
Numan	EVER REPORT TITA PERSON STLC PERAMINED ALKDAM PROVINSIND REPORT	120
Mouse	EVFAREENMENTITACPECTERTASILCHARADLCPALKDAVEELNASNDRCIDVVRNKI	115
Rat		0
Drosophila	SVFATQENAENIIIACPPCVGKTTTIQCHARIHLCDSYKEAVLEINASNERGIDVVRNKI	100
C.elegans	KVIGHEGNVENIVLSEPECCGKTTSVWALARELLEDKVKEAVLELNASDERGIDVVRHRI	94
S.cerevisiae	QQIAKDGNMEHMIISGMPGIGKTWSVHCMAHEDIGRSYADGVLELNASDDRCIDVVRNOT	93
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Human	AN FACENET DESCRIPTION DE LO D	175
Rouse	ILDEADSMD GAOOALRETME IYSKTTRFALACNASOKI IEP	42
Drosophila	KMPAOCKVTHERGEHKTVTLDEADSMTEGAQQALERTMEIYSSTTEFALACNTSEKI LEP	160
C.elegans	KTFAOAKVTLPEGRHKIIIILDEADSMTDGAQQALRRTMEMYTKTTRFALACNOSEKIIEP	154
S.cerevisiae	KHFAOKKLHLEPGKHKIVILDEADSMTAGAQQALRRIMELVSNSTRFAFACNOSNKILEF	153
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Human	TO SREAVERT KET DAOULTRINKVI EKSKVPYTDDOLEDALI UTAGOMONI AND ST	235
Mouse	TO SREAT INTITION OVER LAND TERSKY PYTDDE ANALISWA OF DECALANI OSAF	102
Broscobila	TOSECAM PETKISDAOVLAKLIEVAKWEKLNYTEDGLEAIVETACGDMR9GLNNL9STA	220
C elegans	TOSEGAL HEYTKI SPVOLLTRVKEVAKAEKVNYDDGGHEAHLEWACGDMRCALNNLOATV	214
S.cerevisiae	LOSRCATERYSKESDEDVLKRLLQIIKLEDVKYTNDGLEAIIFTAEGDMRCAINNLOSTV	213
		200
Human	SGFGFINSENWEN/CEEPHILL/KESIOHCVNANIDEA/KHLARLWHIGISEBJIIGHIS	295
Mouse	SGFGYINSERWERVCREISHELLVREWICHCVDANIDEAYKHLAHLWHLCYSPERVIGNIB	162
Rat	OGFGITASENVERVOEEEHEKLLEEMIHHCAANDIHKAYKULAKLWKLEYSPEDIIANIS	280
Colegans	NAVELUNKENWIKVOREPHODIMIKVIHYCTORKFFEASKLIHEFHRLEFSSDDIVSTLE	274
S.cerevisiae	AGHGLVNADNVFKIVDSPHELIVKKVLLASNLEDSIQILRTDLWKKEYSSIDIVTTSP	271
		254
Human	RVCKTFOMAEYLKLEFIKEIGYTHOKIAEVNSIAUMAGLIARLCOKTMAPVES-	349
Mouse	WOWTFPMAEI LILEPHILENSI THER VALE WISTERSTONS LIGHT CONTRAP VES-	216
Rat	NCWITTPHALI	331
Drosophila	NAME AND STATE AND A STATE AND	334
C.eregans	ENTENLAOVKESVRLEMIKEUGLTUMRILEEVGTYUGLASMLOKIHKLNNKA	323
0.001041010¢		

Fig. 3. A: Complementary DNA sequence alignment of the human (GenBank accession M87338), mouse (AF208499), and rat RFC p40. Our cDNA clone (AF208499) contributes the partial coding sequence and the 3'-UTR up to nucleotide 820, while expressed sequence tags Al232965 and AA899195 contribute the rest of the 3'-UTR. Identical residues are shaded. **B:** Amino acid sequence alignment of RFC p40 (RFC2) from human, mouse, rat, drosophila, *C. elegans*, and RFC4 from *S. cerevisiae*. Identical residues are shaded. RFC boxes II, III, IV, V, VIb, VII, and VIII are conserved among the four small subunits of the RFC complex. RFC box V shows similarity to the DEAD-box proteins that are found in ATPases.

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p140 subunit of the RFC complex showed approximately the same mRNA expression during the same period, except that a consistent dip was observed at day five. This implies that the p140 and p40 genes, though encoding parts of the same RFC complex, are under different transcriptional regulation. This relatively sustained p140 expression, which is not coordinated with p40 down-regulated expression, may implicate roles other than DNA replication. RFC p140 was reported to be involved in 3T3-L1 adipocyte differentiation, and its attenuation, by antisense oligonucleotide inhibited differentiation [Lyle et al., 1996]. Other studies also suggested that p140 may be a transcription factor [Lu et al., 1993; Takeoka et al., 1998]. The possible involvement of p140 in functions other than DNA replication may, in part, help to explain its wavering levels on days five and seven, and also its uncoordinated mRNA expression with p40.

On the grounds of the preferential downregulation of p40 mRNA during neonatal development in rat heart, we speculate that the function of RFC in supporting processive DNA replication may be modulated through the p40 subunit. Further investigation into the regulatory elements of the RFC p40 gene may reveal other players involved in differentiation.

Although the data that we reported here do not account for the amount of protein products and were confined to the early postnatal period, our findings enhance the current understanding on the inhibited proliferation of cardiac myocyte during postnatal development. In addition to the induction of a CDK inhibitor p21 [Flink et al., 1998] and the down-regulated activities of CDKs 2, 4, and 6 [Kang and Koh, 1997] shortly after birth in rat myocardium, the p40 subunit of RFC, which is an essential processive for PCNA-dependent DNA replication, is also reduced at mRNA level. Then it is not surprising that the promotion of G1 CDK activities by serum stimulation in rat neonatal cardiac myocytes resulted in no induction of DNA synthesis [Tamamori et al, 1998]. Whether cardiac myocytes are really terminally differentiated and permanently withdrawn from the cell cycle or not, evidences are accumulating that their cell cycle is impeded at multiple points, including the S-phase DNA synthesis. If we are to regenerate the injured mammalian myocardium, these blocks need to

be released one by one unless a cardiomyogenic determining factor is found.

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