ARTICLES

Analysis of the Capacity of Extracts From Normal Human Young and Senescent Fibroblasts to Support DNA Synthesis In Vitro

W.R. Pendergrass,[1]* M. Gray,[1] M.S. Wold,[2] P. Luo,[1] and T.H. Norwood[1]

[1]Department of Pathology, University of Washington, Seattle, Washington 98195–7470
[2]Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242–1109

Abstract   Cytoplasmic extracts from early-passage (young), late-passage (senescent) normal human fibroblast (HF) cultures and immortalized human cell lines (HeLa, HT-1080, and MANCA) were analyzed for their ability to support semiconservative DNA synthesis in an in vitro SV40-ori DNA replication system. Unsupplemented extracts from the three permanent cell lines were demonstrated to be active in this system; whereas young HF extracts were observed to be minimally active, and no activity could be detected in the senescent HF extracts. The activity of these extracts was compared after supplementation with three recombinant human replication factors: (1) the catalytic subunit of DNA polymerase α (DNA pol-α-cat), (2) the three subunits of replication protein A (RPA), and (3) DNA topoisomerase I (Topo I). The addition of all three recombinant proteins is required for optimum activity in the young and senescent HF extracts; the order of the level of activity is: transformed > young HF > senescent HF. Young HF extracts supplemented with RPA alone are able to support significant replicative activity but not senescent extracts which require both RPA and DNA pol-α-cat for any detectable activity. The necessary requirement for these factors is confirmed by the failure of unsupplemented young and senescent extracts to activate MANCA extracts that have been immunodepleted of DNA pol-α-cat or RPA. Immunocytochemical studies revealed that RPA, DNA pol-α, PCNA, and topo I levels are higher in the immortal cell types used in these studies. In the HF cells, levels of DNA pol-α-cat and PCNA are higher (per mg protein) in the low-passage than in the senescent cells. By contrast, RPA levels, as determined by immunocytochemical or Western blot studies, were observed to be similar in both young and senescent cell nuclei. Taken together, these results indicate that the low to undetectable activity of young HF extracts in this system is due mainly to reduced intracellular levels of RPA, while the senescent HF extracts are relatively deficient in DNA polymerase α and probably some other essential replication factors, as well as RPA. Moreover, the retention of RPA in the senescent HF nuclei contributes to the low level of this factor in the cytoplasmic extracts from these cells. J. Cell. Biochem. 73:176–187, 1999.
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The limited proliferative capacity or senescence of cultured normal human fibroblasts (HF) is now a well-established phenomenon [for reviews, see Norwood and Pendergrass, 1992; Cristofalo and Pignolo, 1993; Campisi, 1996; Smith and Pereira-Smith, 1996]. Studies showing that telomere length may be the primary determinant of the proliferative capacity of these cells have recently been published [Bod-

nar et al, 1998]. If telomere loss is confirmed as the biological clock for the division potential of these cultures, the next experimental objective will be to elucidate the mechanism(s) by which telomere length is translated into a growth inhibition signal. It has been established that late passage (senescent) cells lose the capacity to initiate DNA synthesis. This conclusion is supported by the observations that (1) most senescent cells display a G1 DNA (2C) content [Schneider and Fowlkes, 1976; Yanishevsky et al., 1974], (2) the capacity to induce certain genes before the onset of DNA synthesis with serum stimulation [Pendergrass et al., 1991a,b; Afshari et al., 1993; Lucibello et al., 1993], and (3) DNA synthesis can be reinitiated in the

postmitotic cells after infection with the SV40 virus [Gorman and Cristofalo, 1985] or fusion to some transformed cell types [Norwood et al., 1975; Pendergrass et al., 1991a].

The elucidation of the mechanism(s) leading to the loss of the capacity to enter the S phase has been a central focus of research in cellular aging in vitro. On the basis of indirect experimental evidence, a number of investigators have postulated the induction and/or activation of an inhibitor of DNA synthesis in these senescent populations [Smith and Lumpkin, 1980; Rabinovitch and Norwood, 1980]. Recently p21$^{sdi1/waf1}$, an inhibitor of cyclin-dependent kinases (CDK), was cloned from a senescent cell library by Smith and colleagues [Noda et al., 1994]. The inactivation of the cyclin E/cdk2 complex by p21$^{sdi1/waf1}$ apparently prevents the induction of critical components of the DNA replication complex, preventing the initiation of chromosomal replication [Nakanishi et al., 1995]. Recent studies have shown that inactivation of p21 by sequential homologous recombination results in an extension of proliferative capacity apparently bypassing "normal" senescence [Brown et al., 1997]. However, the mechanism(s) that regulate this or any other inhibitor in senescent cells remain to be determined.

Suppression of the induction of critical DNA replication factors in senescent cells could provide a proximate mechanism for the failure of senescent cells to enter the S phase. However, little is known about the functional status of the components of the DNA replication complex in senescent cells. This lack of knowledge reflects in part the paucity of information regarding the molecular mechanisms of the regulation of DNA replication in mammalian systems. The large T antigen-dependent in vitro DNA synthesis system, first described by Li and Kelly [1984], has provided an important experimental approach to the identification of factors involved in the initiation of DNA replication in mammalian cells [Tsurimoto and Stillman, 1989; Tsurimoto et al., 1990; Brush et al., 1995]. In addition to T antigen, seven cellular proteins are required for SV40 replication in vitro. These include DNA polymerase α/primase, a complex of four subunits that can initiate and carry out DNA replication; replication protein A, a three-subunit complex that binds tightly to single-stranded DNA; and topoisomerase I, a type one topoisomerase that is required to remove topological links between the two strands of the parental DNA helix during replication [Stewart et al., 1996]. In addition, topoisomerase II is required for proper release of unit length of the plasmid template and DNA polymerase δ, DNA ligase I, replication factor C, and RNase H are required for complete semiconservative replication [Brush et al., 1995].

In this report, we describe studies investigating the capacity of cytoplasmic extracts prepared from extracts from late-passage (senescent), and low-passage (young) normal human fibroblast cultures, as well as immortalized cell lines (HeLa, HT-1080, and MANCA), to support semiconservative DNA replication in this system. We observed minimal or undetectable levels of activity in cytoplasmic extracts from both young and senescent HF cultures. By contrast, extracts from transformed cell types were shown to be active in this system, as has been previously reported. Supplementation of old and young HF extracts with specific recombinant replication factors, known to be essential for T-antigen-dependent replication of SV40-ori containing plasmids, resulted in significant stimulation of semiconservative replication supported by HF extracts. The general conclusion arising from these studies is that the low activity of the cytoplasmic HF extracts compared with those from the immortalized cells is largely due to a reduced level of critical replication factors and that some, but not all, of these factors are further reduced in senescent calls.

MATERIALS AND METHODS
Cells and Cell Culture

The normal human foreskin fibroblast line, used in these studies, was established in our laboratory from a clinically normal newborn infant. All cultures were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml streptomycin, and 50 μg/ml penicillin. This cell line displays a maximum life span of 65–70 population doublings (PD). Senescent cultures were harvested at 65–70 PD (24 h [^{3}H]thymidine labeling index 10%), and the young cultures at 25–35 PD (24 h [^{3}H]thymidine labeling index ≤85–95%), for preparation of the extracts. MANCA, an established human B-cell line, was provided kindly by James Roberts, and the HeLA and HT-1080 cell lines used in our laboratory have previously been described [Pendergrass et al., 1985].

Reagents and Probes

The monoclonal antibody, 71 [Erdile et al., 1990], and the mouse monoclonal, RPA32–1 [Kenny et al., 1990], were used to identify the 32-kDa subunit of replication protein A (RPA). The murine monoclonal antibody, 70 [Kenny et al., 1990], was used as the probe for the 70-kDa subunit of this replication complex. DNA polymerase α catalytic subunit (DNA pol-α-cat) was probed with the monoclonal antibody, 132–20 [Wong et al., 1986]. Polyclonal rabbit anti-human topoisomerase I (Topo I) antibodies [Stewart et al., 1996] and purified recombinant human Topo I were kindly provided by Dr. James Champoux. The active form of RPA consists of three subunits that were co-expressed in *Escherichia coli* from the transfected plasmid p11dtRPA and were purified as described by Henricksen et al. [1994] from cultures of *E. coli* that had been transfected with the plasmid p11dtRPA.

Immunoaffinity Purification of Human DNA Polymerase α and SV40 Large T Antigen

Pure preparations of recombinant DNA pol-α-cat and large T antigen were prepared by immunoaffinity chromatography from extracts of sf9 cells that had previously been infected with the retroviral constructs AcHDPa (for DNA pol-α-cat)[Copeland and Wang, 1991] or 941T (for the large T-antigen)[Parsons et al., 1990]. The monoclonal antibodies SKJ237 to DNA pol-α-cat [Copeland and Wang, 1991] and AB108 to the large T antigen [Parsons et al., 1990] covalently linked to α-Sepharose as described by Dixon and Nathans [1985] were used in the immunoaffinity purification of these factors. The purification of DNA pol-α-cat were carried out as described by Wong et al. [1986] and for the T antigen as described by Dixon and Nathans [1985]. The purity of the T antigen was assessed by Western blot analysis and the functional capacity of the chromatographed material by its capacity to stimulate DNA replication in a standard MANCA extract. The functional status of the purified DNA pol-α-cat was determined via an activity assay using activated calf thymus DNA as a template [Pendergrass et al., 1991a].

Cell Extracts

The cell extracts used in these studies were prepared using a modification of the method of Li and Kelly [1984]. For production of the young or senescent cell extracts, 150-cm^2 flasks were plated at a density of $\sim 5 \times 10^5$ cells/flask in medium supplemented with 0.2% FBS for 3 days and then stimulated with 10% FBS-supplemented medium and maintained for 44 h before harvesting. At the time of harvest, most of the young cells are in the second S-phase after stimulation, while most of the senescent cells fail to initiate DNA replication but display maximal induction of DNA polymerase α [Pendergrass et al., 1985]. We have observed that the cytoplasmic extracts from cultures in the first S-phase after serum stimulation exhibit lower replicative activity in this system and lower DNA pol-α activity. HeLa and HT-1080 cells were also plated at 5×10^5 cells/T150 flask and allowed to grow for 3 days before harvest while in logarithmic growth. The MANCA cells were grown in suspension cultures and were also harvested while in logarithmic growth at a cell density of $\sim 5 \times 10^5$ cells/ml. The harvest was initiated by trypsinization (of the substrate-dependent cultures) and centrifugation at 1,000g for 5 min. After centrifugation, an equal volume of hypotonic buffer (20 mM HEPES buffer, pH 7.5; 5 mM KCl; 1.5 mM MgCl$_2 \cdot$ H$_2$O; 1 mM DTT) was added to the cell pellet and the cells allowed to swell for 60 min on ice with occasional agitation. The swollen cells were lysed by homogenization with a Dounce homogenizer, using a tight pestle. The extent of lysis was monitored by phase-contrast microscopy and considered complete when $\sim$80% of the cells appear as free nuclei, usually requiring $\sim$10 strokes. The homogenate (the cytoplasmic extract) was adjusted to 0.1 M NaCl and homogenized for two more strokes and spun at 100,000g in a Beckmen 50.1 rotor for 60 min. The clear supernatant was then removed and aliquots stored in liquid N$_2$ for future studies.

In Vitro Replication Assays

A modification of the method of Li and Kelly [1984] was used to assay the level of DNA replication supported by the extracts. The double-stranded plasmid M13 amp SV2, which contains a complete SV40 replication origin, was used in these assays [Roberts and Kunkel, 1988]. The reactions were carried out in 25 µl of a reaction mixture that consisted of 50 ng of template DNA (M13mpSV2), 1 µg/ml of affinity-purified T antigen, 30–100 µg cell extract protein, 100 µM dNTPs, 200 µM rNTPs, 30 mM

HEPES buffer (pH 7.5), 7 mM $MgCl_2$, 4 mM ATP, 40 mM phosphocreatinine, 100 µg/ml phosphocreatinine kinase, and 200 µCi/ ml [³H]dTTP. The reaction was allowed to proceed for 60–180 min at 37°C and the extent of [³H]dTTP incorporation determined by scintillation spectroscopy. The evaluation of the extent of semiconservative DNA replication of the template was assessed by the determination of the extent of the acquisition of resistance to *Dpn*I digestion due to loss of methylation 5′-GATC-3′ sites after replication of the M13mp2SV plasmid in vitro [Roberts and Kunkel, 1988]. *Stu*I was included in the digestion to linearize the plasmid at a site near the origin of replication. Resolution of the ³²P-labeled products of the digestion was accomplished by electrophoresis in 1.0% agarose with visualization by autoradiography.

Depletion of Pol α and RPA from MANCA Extracts

The depletion of specific factors from this cellular extract was accomplished by precipitation with specific antibodies. The murine monoclonal anti DNA polymerase α antibody, 132–20 [Wong et al., 1986], or anti RPA, 71 [Erdile et al., 1990] were used in these depletion studies. To 400 µl of MANCA extract, 3.5 µg anti DNA pol-α or 7 µg of anti RPA was added and the mixture allowed to stand for 20 min at room temperature to permit binding. Then the extract was transferred to a microfuge tube containing 150 µl of a suspension of swollen Sepharose beads covalently linked to anti-mouse IGG (Cappel, Durham, NC). After incubation for ~20 min to permit antibody binding, the beads were pelleted and depleted cytoplasm (supernatant) removed, aliquoted, and stored in liquid nitrogen. A sham depletion (with no primary antibody) was carried out for control studies.

Immunocytochemical Studies

The cultures to be stained were rinsed with phosphate-buffered saline (PBS) before fixation in 2% formalin in PBS for 10 min and then stored at − 20°C. The fixed cells were then blocked with 3% bovine serum albumin (BSA) and 20% goat serum for 60 min at 37°C before the addition of primary murine anti-RPA (71) at a 1:300 dilution or anti-topoisomerase I at 1:500. After a 30-min incubation at 37°C, the preparations were washed with PBS three times before incubation for another 30 min with a 1:200 dilution of a biotinylated secondary antibody:

goat anti-mouse for RPA, or goat anti-rabbit for Topo I. Finally, the antigen-antibody complexes were visualized using an ABC horseradish peroxidase (HRP) staining kit from DAKO (Carpinteria, CA). Control slides were exposed to an isotypic primary antibody and then processed as described above. PCNA was stained using EPOS reagent kit containing murine anti-PCNA antibody linked directly to HRP.

Immunoblot Studies

Western blot studies were carried out using the methods recommended by Boehringer Mannheim (Indianapolis, IN). The cell pellets were lysed in 1% sodium dodecyl sulfate (SDS), boiled for 5 min and then mixed with the electrophoresis buffer (0.125 M Tris-HCl [pH 6.8]), 10% glycerol, 10% β-mercaptoethanol, 0.02% bromophenol blue dye; the electrophoresis was run on 15% SDS-polyacrylamide gel electrophoresis (PAGE) gels with molecular-weight standards. After electrophoretic transfer to a nitrocellulose membrane, these blots were stained with the antibodies to the specific antigen and visualization was accomplished by exposure to a biotinylated secondary antibody, followed by reaction with avidin containing HRP, and finally a chemiluminescent substrate.

RESULTS
Assay of Cell Extracts in the SV40 System

Extracts from the transformed cells were all observed to support the incorporation of 15–40 pmoles/assay during a 3-h incubation at 37°C. The highest level of activity was consistently observed in the MANCA cell extracts, which were used in the majority of studies comparing the activities of HF and transformed cell extracts (Table I). By contrast, the incorporation of <1 pmole dTTP/assay was observed in reactions with extracts from both young and senescent HF.

We then examined the effect of supplementing the extracts with purified recombinant proteins including: DNA pol-α-cat, RPA (holocomplex), and Topo I (Table I). These factors have been found necessary for complete replication of the SV40 ori template [Tsurimoito et al., 1990]. As indicated above, DNA polymerase α is known to be required for chromosomal DNA replication [Stillman, 1996; Brush et al., 1995], RPA binds to single-strand DNA in the replication bubble and is thought to be involved in the

TABLE I. Extent of Incorporation of [³H]TTP into the Template Plasmid, M13mpSV2, in Representative Studies With Cytoplasmic Extracts from the Cell Types Used in These Studies With and Without Supplementation With Selected Replication Factors[a]

Cell type	DNA Polα cat (units)	Supplementation		[³H]TTP incorporated[b,c] (pmole/µg protein)	Total (pmole)
		RPA (ng)	TopoI (ng)		
MANCA	—	—	—	0.22	35
MANCA	0.6	450	25	1.29	95
Young HF	—	—	—	<0.02	0.04
Young HF	—	450	—	0.08	3.5
Young HF[c]	0.6	375	—	0.2	9
Young HF	0.6	450	25	0.44	32
Senescent HF	—	—	—	<0.02	0.3
Senescent HF	—	375	—	0.02	0.8
Senescent HF	0.6	375	—	0.05	2
Senescent HF[d]	0.6	450	25	0.25	9

[a]Controls for all studies included reaction minus T antigen (>0.01 pmoles/µg extract protein incorporated in 3 h), reaction with three supplemental factors and T antigen, but no cytoplasmic extract (~0.41 pmoles [³H]TTP was incorporated in 3 h.
[b]All reactions were incubated for 3 h.
[c]The amount of protein in the extracts varied from preparation to preparation; the amount added to each reaction mixture varied from 40 to 100 µg. The reaction is approximately linear over the concentration range.
[d]These reactions were carried out with cytoplasmic extracts from a different preparation than was used in the other three reactions. We have consistently observed no greater than a two-fold variation in the extent of incorporation in these reactions.

stabilization of that structure [Kenny et al., 1990; Henricksen et al., 1994]; Topo I is involved in the regulation of the topography DNA during replication and other metabolic processes [Champoux, 1994; Stewart et al., 1996]. The addition of 125–450 ng of RPA alone stimulated the incorporation of 3–4 pmoles in reactions with young cell extracts but was observed to be minimally stimulatory when added to senescent extracts (<1.0 pmole). The supplementation of MANCA extracts with RPA holocomplex also resulted in a significant increase in precursor incorporation. By contrast, the addition of DNA pol-α-cat or Topo I alone did not stimulate replicative activity in any of the extracts used in these studies (data not shown). The combined addition of DNA pol-α-cat and RPA stimulated a modest increase in precursor incorporation with senescent extracts and a marked increase in this activity was seen when the reaction mixture was supplemented with all three of the factors. The young cell extracts consistently supported a level of activity three- to fivefold higher than the old cell extracts. No activity could be detected in the absence of a cytoplasmic extract, even when all three recombinant proteins and T antigen were present in the reaction mixture.

A significant portion of the replication products produced in the reaction with only supple-mentation were observed to be resistant to DpnI digestion, consistent with the occurrence of semiconservative replication (Fig. 1A,B). The relative amount of product resolved in these gels was determined by measurement of the integrated optical densities of the bands. The estimates by this method (using DpnI digestion) showed that the extent of replication in the senescent and young cell extracts supplemented with all three factors (RPA, pol-α-cat, and Topo I) were 5% and 40%, respectively, of similarly unsupplemented MANCA. The values for incorporation of ³²P into DpnI-undigested material relative to MANCA controls in Figure 1A are roughly similar to those shown in Table I. The variations observed are probably the result of slightly varying replication conditions, especially the 90-min replication time used for the gels compared with the 3-h time used in the studies shown in Table I.

Depletion and Mixing Studies

In order to determine whether DNA pol α and/or RPA are rate-limiting components in the reaction in the fibroblast extracts, we investigated the capacity of these extracts to complement MANCA extracts that had been immunodepleted of these factors. The immunodepletion of RPA reduced the replicative activity of the MANCA extracts to undetectable levels (Table
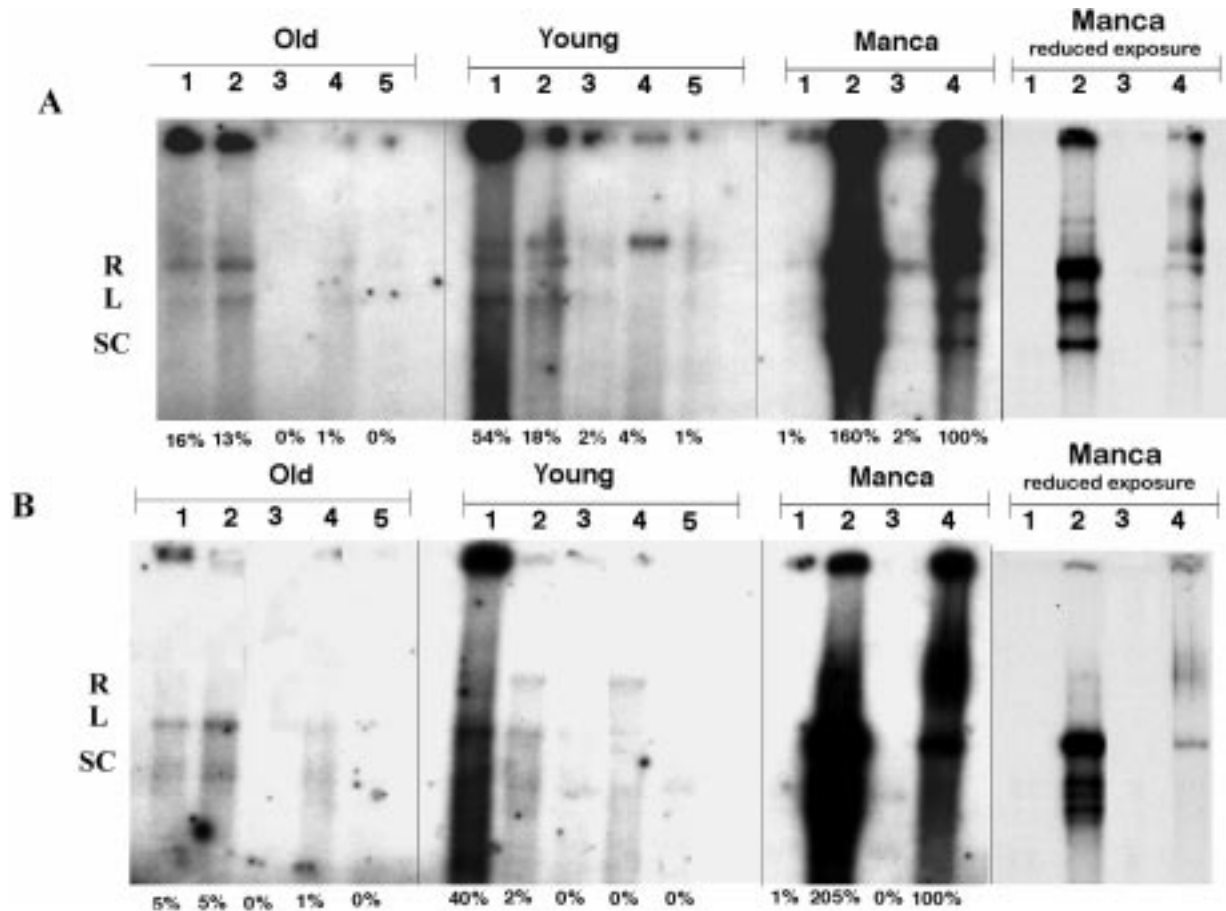
Fig. 1. Product analysis gels of M13mpSV2 after large T-antigen-dependent replication in the presence of old or young fibroblast or MANCA cell extracts supplemented with various combinations of replication factors without **(A)** or with **(B)** digestion with *Dpn*I and *Stu*I as described under Methods. The old and young cell extracts (72 µg protein) were supplemented with replication factors as follows: 0.72 U DNA pol-α-cat, 450 µg RPA (all subunits), 25 ng Topo I (**lane 1**); DNA pol-α-cat and RPA (same concentrations) (**lane 2**); Topo I only (**lane 3**); RPA only (**lane 4**); and DNA pol-α-cat only (**lane 5**). The reaction mixtures were incubated for 90 min and the plasmid replication product isolated as described under Materials and Methods. In the panel labeled MANCA, lanes 1 and 3 were loaded with 66 µg of extract protein without large T antigen; the reaction mixture loaded into lanes 1 and 2 was supplemented with the three replication factors at the same concentrations described above. The values at the bottom of each lane indicate the percent of the sum of the optical densities of the relaxed, linear, and super-coiled bands observed in the unsupplemented MANCA reaction (lane 4, MANCA panels). The autoradiograms shown in the

fourth figure in A and B were exposed for a shorter period to demonstrate more clearly the replication products from the incubation with the MANCA extract that are being resolved. The location of the relaxed (R), linear (L), and supercoiled (SC) bands are indicated at the left side of A and B. The percentage values shown at the bottom of each lane indicate the extent of DNA replication, as assessed by measurement of the integrated optical density, relative to that occurring in the reaction with unsupplemented MANCA extracts. The band evident in the undigested preparation (A) immediately proximal to the relaxed material and especially prominent lane 4 in the gels of the young HF and MANCA is thought to be a replication intermediate. The large amount of high-molecular-weight reaction product, particularly evident in the autoradiograms of the senescent and young HF gels, is thought to be caused by rolling circle replication, which has been previously reported to occur in this in vitro DNA synthesis system [Li and Kelly, 1985]. An increase in rolling circle replication could be related to the low levels of some key replication factors in the young and old HF extracts (M.S. Wold, unpublished communications).

II). Supplementation of the depleted extracts with recombinant RPA restored the activity to baseline levels or higher, consistent with the specific depletion of this factor. However, the addition of extracts from young or old fibroblasts did not result in any detectable increase in the replicative activity in these RPA depleted extracts, consistent with the presence of very low levels of RPA in both HF extracts. The immunodepletion of DNA pol-α from the MANCA extract resulted in a reduction of ~80% of its activity in the SV40 system (Table III) and ~90% of the activity of this polymerase in the extract (data not shown). Supplementation of

TABLE II. Depletion and Reconstitution of RPA in MANCA Cell Cytoplasmic Extracts

Condition[a]	RPA suppl	pmoles [³H]TTP incorporated/µg protein[b]
Depleted	—	0.72 ($<$0.02)
Mock depleted[c]	—	42 (0.47)
RPA suppl[d]	187	37 (0.38)
RPA suppl[d]	375	51 (0.58)
RPA suppl[d]	500	144 (1.64)
Young HF extract suppl[e]	—	$<$0.02
Senescent HF extract suppl[e]	—	$<$0.02

[a]A total of 88 µg of protein was added to the reaction mixture in all these studies.
[b]The reaction was allowed to proceed for 3 h.
[c]In the mock depletion, all steps of the procedure were carried out in the absence of the RPA antibody.
[d]Supplementation to depleted extracts.
[e]A total of 8 µl of young or senescent HF cytoplasmic extracts was added to these reaction mixtures.

TABLE III. Depletion of DNA Polymerase α and Readdition of DNA pol-α-cat to MANCA Extracts[a]

Condition	Pol-α-cat (units)	pmoles [³H]TTP incorporated/µg protein[b]
Depleted	—	5.0 (0.06)
Mock depleted[c]	—	42.0 (0.48)
Pol-α-cat suppl[d]	1.2	13.0 (0.15)
Pol-α-cat suppl[d]	2.0	11.0 (0.13)
Pol-α-cat suppl[d]	3.2	9.0 (0.1)
Young HF extract suppl[e]	—	4.7 (0.02)
Senescent HF extract suppl[e]	—	1.0 (0.02)

[a]88 µg of MANCA cytoplasmic extract added to each reaction.
[b]The reaction was allowed to proceed for 3 h.
[c]In the mock depletion, all steps of the reaction were carried out in the absence of the Polα antibody.
[d]Supplementation of depleted extracts.
[e]A total of 8 µl of young or senescent HF cytoplasmic extracts was added to these reaction mixtures.

these depleted extracts with recombinant DNA pol-α-cat resulted in only partial restoration of replicative activity to these depleted extracts; again, the addition of extracts from young or old fibroblasts did not result in a discernible increase in their capacity to support DNA replication. It should be noted that depletion of DNA pol-α-cat with specific antibody also removes the polymerase holoenzyme containing pri-

mase and other subunits not replaced by addition of recombinant catalytic subunit.

Immunocytochemical Studies

Immunocytochemical studies were carried out in an effort to determine whether the observed differences in the levels of activity in the unsupplemented extracts could be related to inherent differences in the intrinsic levels of the factors in the various cell types. Staining with antibodies to the 32-kDa or the 70-kDa subunit of RPA showed that this factor is almost exclusively confined to the nucleus; the most intense signal was observed in the serum stimulated HT-1080 cells with less intense staining evident in the nuclei of young or senescent HF (Fig. 2). The intensity of staining in the senescent nuclei appears to be slightly greater than that seen in the young nuclei. Other immunostaining studies with antibodies to Topo I, pol-α, and PCNA demonstrated the most intense signal in the HT-1080 nuclei > young nuclei > senescent nuclei. Only an occasional senescent cell faintly stained with the DNA pol-α or the PCNA antibodies was seen in these preparations. Topo I demonstrated a stronger signal than PCNA or pol-α, in senescent nuclei but was still obviously lower than that observed in the young nuclei.

Immunoblot Analyses of the Cellular Extracts

Because the supplementation of the young and senescent cell extract with RPA resulted in the most significant enhancement of replicative activity, we decided to obtain a more quantitative estimate of the levels of this factor in cytoplasmic and whole cell extracts via Western blot analysis (Figs. 3, 4). Estimates of the amount of RPA present in the 25-µl reaction mixture (Table IV, column 5) indicated that the MANCA extracts contained four to five times the reaction mixtures prepared with the young cytoplasmic extract. The mixtures containing young HF extracts contained approximately the same amount of this factor as the RPA-immunodepleted MANCA extract, while the reactions carried out with senescent HF extract contained only 5–10% of the RPA levels present in those with the young HF extract. These values are consistent with the levels of activity of the various cytoplasmic extracts observed in the in vitro assays, assuming that RPA is rate limiting. We also observed that two bands were consistently present in the senescent HF cyto-
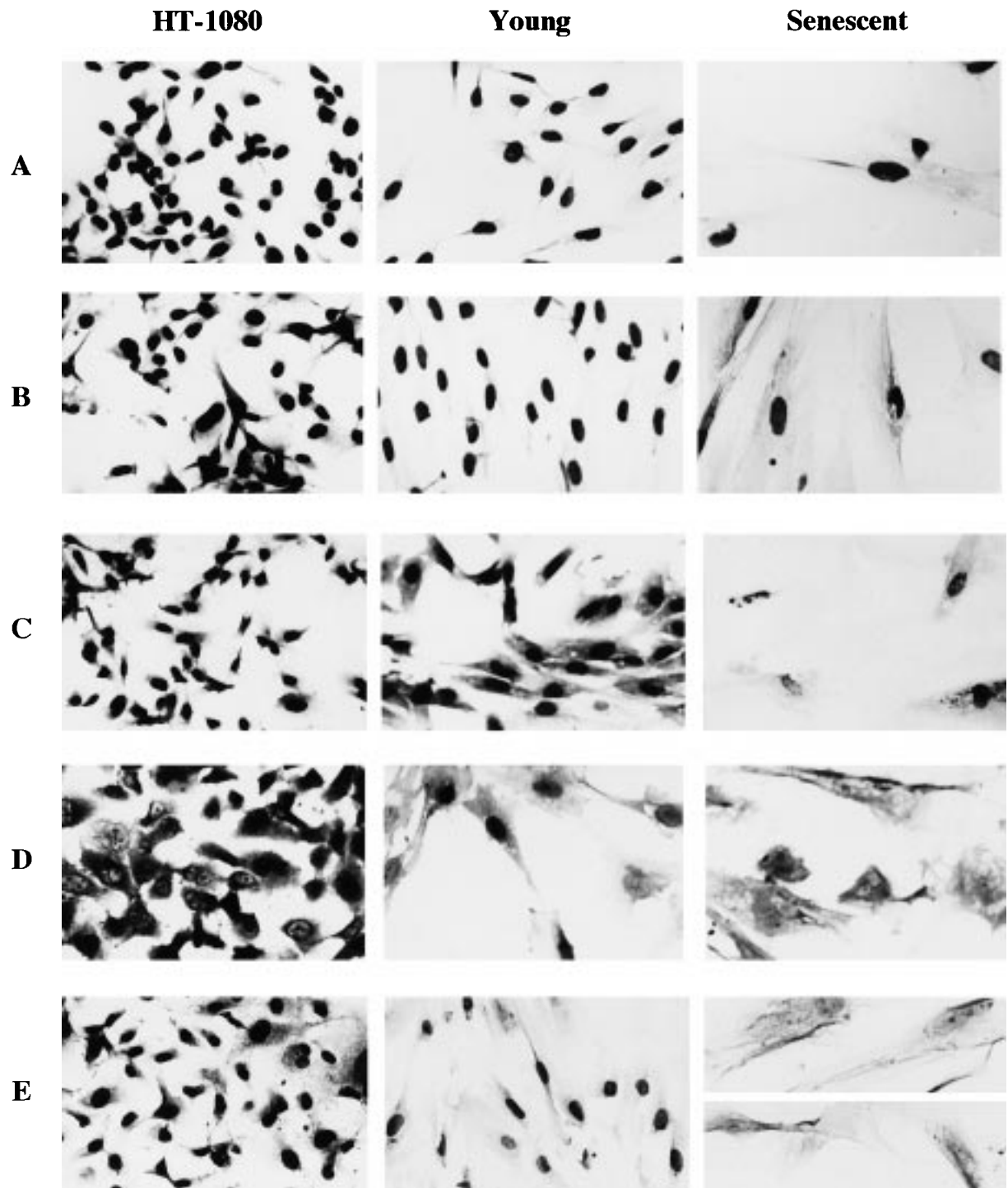
Fig. 2. Photomicrograph of immunocytochemical stains of HT-1080 cells young HF and senescent HF. These cells were stained for the 32-kDa subunit of RPA **(A)**, 70-kDa subunit of RPA **(B)**, Topo I **(C)**, PCNA **(D)**, and DNA polymerase α **(E)**. The methods and antibodies used in these studies are described under Materials and Methods. × ~300.
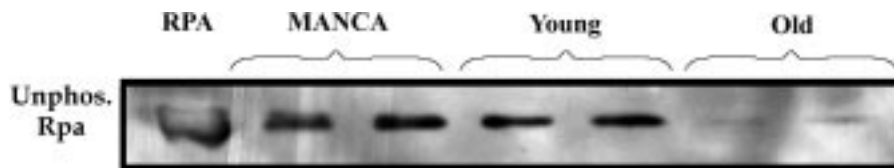
Fig. 3. Western blot analyses of the 32-kDa subunit of RPA in cytoplasmic extracts from two separate MANCA cell preparations: young HF and senescent HF preparations. A standard of 300 ng of recombinant RPA protein was run in the lane indicated. The amount of extract protein loaded into each lane is indicated in Table IV. These studies were carried out using the antibody, 71–9a, to the 32-kDa subunit of RPA.
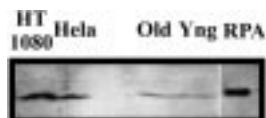

Fig. 4. Western blot analysis of the levels of 32-kDa subunit of RPA in whole cell extracts prepared from HT-1080 cells (**lane 1**), HeLa cells (**lane 2**), blank (**lane 3**), senescent HF (**lane 4**), young HF (**lane 5**), and 125 ng RPA standard (lane 4). These studies were carried out using the antibody, 71–9a, to the 32-kDa subunit of RPA.

plasmic extracts (Fig. 3); the higher-molecular-weight band is thought to be a phosphorylated variant of this RPA subunit.

In Western blot analyses of the whole cell extracts from HF, a somewhat different result was obtained (Fig. 4). As was seen in the studies with the cytoplasmic extracts, the levels of RPA was observed to be highest in the extracts prepared from the immortal cell types (HeLa and HT-1080). However, the calculated levels of this factor were seen to be virtually identical in the whole cell extracts from young and senescent HF (cf. column 4 in Table V and column 4 in Table IV). Similar levels of RPA were evident (by eye) in young and senescent nuclei in the immunocytochemical preparations. We believe that these results are indicative of the retention of a tightly bound and/or an insoluble form of RPA during the preparation of the cytoplasmic extracts from the senescent HF.

DISCUSSION

In these studies, we have shown that, in contrast to cytoplasmic extracts from immortalized cell types, extracts from young and senescent normal HF support minimal, if any, replicative activity in the SV40, large T-antigen-dependent in vitro DNA synthesis system. The supplementation experiments taken together with the immunocytochemical and Western blot studies indicate that the minimal activity exhibited by the HF extracts is due to lower levels of replication factors, especially RPA, in the intact

HF cells. The lower activity observed in the senescent extracts, relative to that observed in the young extracts, even after supplementation, suggests that other replication factors are specifically deficient and/or the presence of an inhibitor (see below) in senescent cells. A 10-fold dilution of the cellular soluble constituents occurs during preparation of the extracts which may account for the suboptimum levels of some of the replication factors in the young and senescent HF cytoplasmic extracts. If one assumes that the levels of these replication factors in the young HF more closely reflect the status of these factors in normal somatic cells in vivo, the high level of activity in the transformed cell extracts used in these studies implies that these factors are present in supernormal levels in these cells.

These results are also consistent with the presence of a specific deficiency of DNA polymerase α in senescent cells. Young HF extracts supplemented with RPA alone were quite active in the SV40 assay, whereas senescent cells supplemented in the same way were inactive unless DNA pol-α-cat was also added (Table I). By contrast, the addition of DNA pol-α-cat to unsupplemented or to RPA supplemented young HF extracts had very little effect.

The immunocytochemical studies showed lower levels of DNA polymerase-α, PCNA and Topo I in the senescent cells. Lee et al. [1997] have also reported reduced levels of Topo I in senescent HF. By contrast, levels of RPA in the senescent extracts were observed to be only slightly reduced relative to the young HF extracts. On the basis of the following studies, we have suggested that DNA polymerase-α may be a rate-limiting factor for the initiation of DNA synthesis in senescent cells: (1) a failure of senescent HF cells to complement a polymerase-α-deficient mutant in heterokaryon studies with a series of temperature-sensitive mutants [Pendergrass et al., 1994]; and (2) that the rate of entry into the S phase in senescent nuclei is

TABLE IV. Quantitation of the Levels of the 32-kDa Subunit of RPA Resolved in Western Blot Studies of Cytoplasmic Extracts from the Various Cell Types

Cell type	(1) Lane[a]	(2) Relative optical density (OD) of band (arbitrary units)	(3) Protein loaded/lane	(4) RPA conc.[b] (ng RPA/µg total protein)	(5) RPA/25-µl reaction mixture[c]
MANCA	2	20	20	3.0	359
MANCA (immunodepleted)	3	14	55	0.8	92
Young HF (prep. 1)	4	16	28	1.7	86
Young HF (prep. 2)	5	14	30	1.4	70
Senescent HF (prep. 1)	6	2	31	0.2	6
Senescent HF (prep. 2)	7	1	29	0.1	3
RPA standard	1	100	—[d]	—	—

[a]Lanes in Western blot gel shown in Figure 3.
[b]RPA concentration relative to total protein in the extract is calculated by the equation:

$$\text{RPA (ng/µg total protein)} = \frac{\dfrac{\text{OD extract}}{\text{OD standard}} \times \text{amount of RPA standard}^{c}}{\text{total extract protein}}.$$

[c]Protein content of MANCA and depleted MANCA was 12 µg/ml; young HF was 5 µg/ml; senescent extracts was 3 µg/ml.
[d]A total of 300 ng of pure recombinant RPA (32-kDa subunit) was loaded into this lane as a standard.

TABLE V. Quantitation of the Levels of the 32-kDa Subunit of RPA Resolved in Western Blot Studies of Whole Cell Extracts from the Various Cell Types

Cell type	(1) Lane[a]	(2) Relative optical density (OD) of band (arbitrary units)	(3) Protein loaded/lane	(4) RPA conc.[b] (ng RPA/µg total protein)
HT-1080	1	46	11	5.2
HeLa	2	92	18	6.4
Young HF	3	20	58	0.25
Senescent HF	4	26	116	0.3
RPA	5	100	—[c]	1,000

[a]Lanes in Western blot gel shown in Figure 4.
[b]RPA concentration determined by the equation in footnote b in Table IV.
[c]A total of 125 µg of pure recombinant RPA (32-kDa subunit) was loaded into this lane as a standard.

directly proportional to the activity level of polymerase-α [Pendergrass et al., 1991a,b]. The DNA polymerase α level is significantly higher in many, if not all, transformed cell types compared with normal fibroblasts [Moore and Wang, 1994].

The reason for the smaller supplement-induced increase in the activity of senescent HF extracts is unclear. Mixing studies between young and cytoplasmic senescent extracts revealed no evidence for a soluble inhibitor in the latter (data not shown). It should be emphasized, however, that inhibitors acting on the transcription, translation or processing of replication factors would not be detected in these mixing experiments. Also, the putative inhibitor could act on a factor(s) essential for chromo-

somal DNA replication in vivo but not required for the initiation of replication of SV40. As mentioned in the opening paragraphs, recent studies suggest that the cyclin-dependent kinase inhibitor, p21, plays a central role in the onset of senescence in cultures human fibroblasts [Brown et al., 1997]. p21 has been shown to inhibit in vitro DNA replication, thought to be mediated by its ability to inactivate PCNA [Luo et al., 1995; Li et al., 1994]. Presumably, the levels of p21 in our senescent cytoplasmic extracts were insufficient to inhibit replicative activity. Assuming that an inhibitor is not present, or is present in insufficient concentrations, in the senescent cytoplasmic extracts, these results suggest that other factors, which are sufficient in the young cells, are decreased

to rate-limiting levels in the senescent cells. PCNA is one possibility; our immunocytochemical studies demonstrated a significant reduction in the level of this nuclear factor, an observation that has been reported by other investigators [Chang et al., 1991; Pang and Chen, 1994]. DNA primase (part of the DNA polymerase α holoenzyme), another factor that is almost certainly essential for activity in this system, has also been reported to be reduced in senescent cells [Collins and Chu, 1990]. To our knowledge, the levels of other factors involved in chromosomal replication in senescent human fibroblasts, such as DNA polymerases δ and ξ, DNA ligase, replication factor C, or protein phosphatase 2A, have not been examined in senescent HF.

Another possible explanation for the failure to completely reactivate senescent cell extracts is the loss of the capacity of these cells to carry out posttranslational modification of factors that are essential for DNA replication in this system. One candidate for such a function is the cyclin E/cdk2 complex, the activation of which is thought to be rate limiting for the progression from the G1 to the S phase in transformed cells [Ohtsubo and Roberts, 1994]. The levels of some of the components in this complex are elevated, but cdk2-associated kinase activity is low or absent in senescent cells [Dulíc et al., 1993]. Supplementation of young and senescent cell extracts with active recombinant cyclin A/cdk2 and cyclin E/cdk2 complexes alone (kindly provided by Dr. Jim Roberts) failed to stimulate increased DNA synthetic activity in these extracts (data not shown).

The decreased level of RPA evident in the Western blots of cytoplasmic extracts almost certainly contributes to the inactivity of both the young and the senescent cell cytoplasmic extracts. Indeed, very significant stimulation of synthetic activity was observed with supplementation of RPA alone. However, this result is apparently not indicative of decreased levels of this factor in the senescent cell nuclei; we observed only a small decrease in the levels of the 32-kDa RPA subunit in whole-cell extracts prepared from senescent cultures as compared with those prepared from young cultures. Similar results were obtained in immunocytochemical staining of young and senescent cells with antibodies to the 32-kDa and 70-kDa subunits of RPA. It may be that these factors are more tightly bound to the matrix of senescent nuclei which could impede the formation of active replication complexes that are required for the initiation and maintenance of chromosomal and/or repair replication in mammalian cells [Applegren et al., 1995]. We have previously shown that the rate of initiation of DNA synthesis is highly inversely correlated with nuclear size [Pendergrass et al., 1991a,b]. It is possible that structural alterations associated with increased nuclear size result in increased binding of certain replication factors to the nuclear matrix.

The results presented in this report suggest that normal HF carry out replicative functions with minimal levels of all, or a subset of, replication factors. The presence of these minimal levels may be a necessary condition for the stringent regulation required for normal cells to be appropriately responsive to extrinsic signals that regulate developmental processes and maintain proliferative homeostasis in adult tissues. Thus, it may be that the capacity of the extracts from transformed cell types to support semiconservative DNA replication in this system is a manifestation of a pathologic alteration in the metabolism of these cells.

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