A 57,000-mol-wt Protein Uniquely Present in Nonproliferating Cells and Senescent Human Fibroblasts

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ABSTRACT Mouse monoclonal antibody, S-30, was produced from hybridoma preparation from mice injected with the cytoskeleton extract of an in vitro aged culture of human fibroblasts derived from a 66-yr-old donor. The antibody stains positively the nuclei of the nonproliferating cells present predominantly in the senescent cultures of five selected fibroblast strains derived from donors of different age groups, whereas a negative reaction is observed in the cultures of their young counterparts. In the intermediate stage of the in vitro life span of these cell strains, a heterogeneous positive reaction for staining with S-30 antibody is observed in different subfractions of cell cultures. However, the expression of S-30 can be induced in the young fibroblasts at the early stage of their life by prolonged culturing to confluence. This induced expression of S-30 nuclear staining can be depleted upon subculturing at low cell density. Immunoelectron microscopy with colloidal gold-protein A complex demonstrates that the S-30 proteins are present in the nuclear plasma and at the region of nuclear envelope in a clustered arrangement. Immunoprecipitation of [^{3}H]leucine labeled cell specimens shows that the antibody S-30 reacts with a protein with a molecular weight of ~57,000.

Cultures of normal human fibroblasts have a finite life span in vitro. This restriction on proliferating capacity has been interpreted as a manifestation of senescence at the cellular level (1, 2). Initially, the cultures proliferate vigorously. However, with successive subcultivation, the growth rate of a culture progressively declines and ultimately falls precipitously. Cristofalo and Sharf (3) have shown that a progressively decreasing fraction of the cells in a population synthesizes DNA, as the population approaches the end of its in vitro life. Senescent fibroblasts that have ceased to proliferate are halted in the G1 phase of the cell cycle (4). Thus, for human fibroblasts, an inability to begin DNA synthesis is a hallmark of cellular senescence.

Although the cessation of replication can be interpreted according to either concept, i.e., cellular senescence vs. terminal differentiation, a common basis exists in that the expression of a new set of genes, governing the cell cycle traverse at the G0/G1 phase of the cell cycle, appears to be needed for the cessation of proliferation to occur. Recent studies of heterokaryons produced by fusion between virus-transformed cells and senescent cultures support this hypothesis and suggest further that the expression of these genes is dominant (5–8). Therefore, near the end of the fibroblasts' in vitro life span, molecular events occur that lead to the production of proteins that may express the cellular commitment to leaving the process of cell cycling. The senescent phenotype may then represent a consequence of these events.

As an approach to the search for the senescence-specific expression of unique proteins, I report here the identification and characterization of a monoclonal antibody that can recognize a protein of 57,000 mol wt in the nuclei of nonproliferating fibroblasts in aged cultures but not in the rapidly dividing cells of their young counterparts.

MATERIALS AND METHODS

Cell Strains: I have used five cell strains, derived from human donors of different ages, to generate fibroblastic cultures at various stages of their in vitro life span. The cell strains received from the Human Genetic Cell Repository (Camden, NJ) were initially incubated in Eagle's minimum essential medium supplemented with 10% fetal calf serum and 1% nonessential amino acid. As soon as cell cultures became confluent, a strict schedule of subcultivation was followed as described (9). In brief, the in vitro life history of a given cell strain can be defined by recording the number of population doublings per passage and the time involved, and the cumulative number of actual population doublings (CPDLs)[1] during passage to the end of the proliferative life span of a culture. The CPDL for each of the five cell strains is listed in Table I.

Preparation of Immunogens: Approximately 1×10^8 cells of

[1] *Abbreviation used in this paper:* CPDL, cumulative number of actual population doublings.

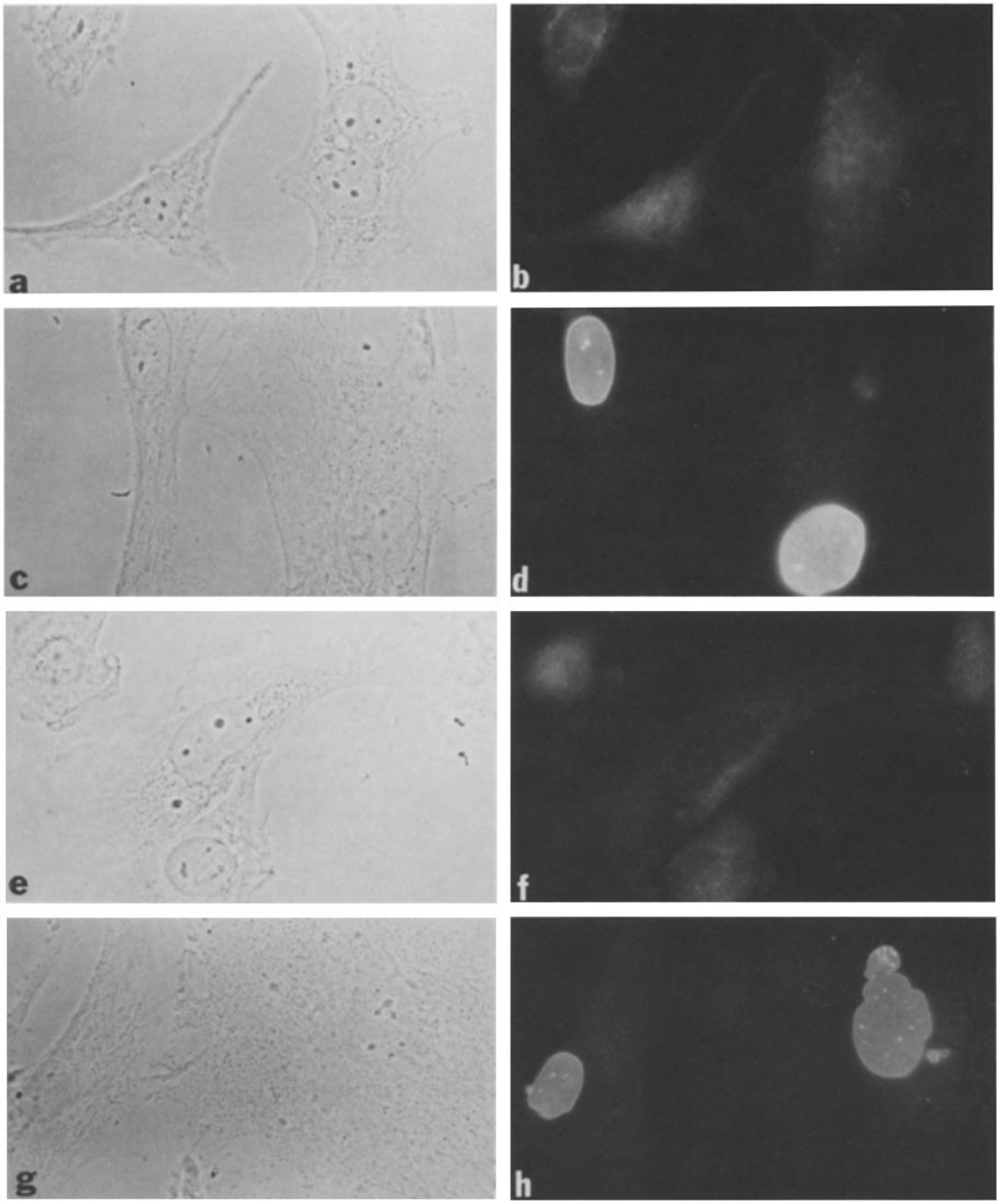

FIGURE 1 The expression of a senescence-specific protein (*SSP*), S-30, in young and old fibroblast cultures of two different cell strains, by phase-contrast (*a, c, e,* and *g*) and immunofluorescence (*b, d, f,* and *h*) microscopy. (*a* and *b*) Lack of S-30 staining activity in the nuclei of 0011 fibroblasts at early stage of their life span (CPDL < 10). (*c* and *d*) The positive staining reaction of S-30 antigen in senescent fibroblasts of the 0011 cell strain (CPDL > 65). Approximately 95% of the cell population at this stage demonstrates the positive nuclear staining pattern shown here. (*e* and *f*) Lack of S-30 staining in young cells of 3529 strain (CPDL < 10). (*g* and *h*) Positive staining reaction of S-30 in senescent fibroblasts of the 3529 cell strain (CPDL > 30). Again, the staining reaction was found in ~98% of the cell cultures. × 600.

nonproliferating senescent cultures of fibroblast strain 3529 (donor age, 66 yr) at CPDL > 30 were used as the starting material. Cells at this stage of life do not show any proliferative activity. These cells were then processed for extraction of cytoskeleton fraction as described (10). The cytoskeletal fraction was then separated into aliquots of protein concentrations (5 mg/ml) and injected as antigens into mice as described (11).

Production and Screening of Mouse Hybridoma: Mouse hybridoma was prepared according to a modification of the procedure originally described by Kohler and Milstein (11, 12). The hybridoma fluid was screened against cells derived from both young (0011, 8 fetal wk) and old (3529, 66 yr) donors at early, intermediate and late CPDLs. The screening was performed directly on formaldehyde-fixed and acetone-extracted cells grown on multiwell microscopic slides as described (13).

Immunofluorescence Microscopy: Cell specimens stained for

monoclonal antibodies were prepared in a way similar to the procedure described for polyclonal antibodies (14), except that the primary antibody was incubated for 12–16 h at 37°C, to ensure binding between immunoglobulin molecules and the cytoskeletal proteins. The second antiserum used for staining monoclonal antibodies was fluorescent goat antimouse immunoglobulin, directed toward IgG and IgM molecules. Goat antimouse IgG was purchased from Cappel Laboratories (Cochranville, PA) or from Antibodies Inc. (Davis, CA). These secondary antisera were absorbed with formaldehyde-fixed and acetone-extracted cell specimens before use in order to remove the nonspecific background reaction. The concentration of goat immunoglobulin used for each staining reaction was standardized to 0.04 mg/ml. Visualization of the prepared specimens was performed with a Zeiss photomicroscope III equipped with epifluorescence illumination and a 63× optic (Carl Zeiss, Inc., Thornwood, NY). The specimens were photographed at ASA 800 with Kodak Tri-X film.

Indirect Immunoelectron Microscopy: Colloidal gold particles, ~5 nm in diameter, were prepared according to the procedure described by Faulk and Taylor (15). These particles were conjugated with protein A (Sigma Chemical Co., St. Louis, MO), and used as described (13). I used an added step of incubation with rabbit antimouse IgG + IgM after the initial staining reaction with the primary monoclonal antibody, before reaction with gold-protein A complex.

Immunoprecipitation: Cell cultures were washed in leucine-free medium and incubated in this medium at 37°C for 15 min before the addition of [^{3}H]leucine (100–200 μCi/ml, 750–1,000 μCi/mmol; New England Nuclear, Boston, MA). Cells were labeled for 10 h in ~5 ml of medium per 100-mm petri dish. Labeled protein fractions were immunoprecipitated by modification of the protein-A method of Kessler (16). In brief, 2 μl of antiserum was mixed with 10 μl of the labeled extract, which had been adjusted to 0.1% SDS and 0.5% Nonidet-P40 (NP40). The mixture was incubated at 4°C overnight. Rabbit antimouse IgG was added to the mixture at a concentration of 0.04 mg/ml and incubated at 4° for 2 h. Antigen-antibody complexes were then precipitated with 40 μl of a 1:10 (vol/vol) suspension of protein-A Sepharose beads. The precipitate was washed three times with 1% NP40, 10 mM Tris-HCl (pH 8.0), and three times with 10 mM Tris-HCl (pH 8.0), as described by Krueger et al. (17). The washed immunoprecipitates were then boiled for 5 min in 0.06 M Tris-HCl, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol, and centrifuged to remove the beads. The supernatant was analyzed by electrophoresis and autoradiography as described (14).

Quantitation of Growth State: The status of cell growth was examined by long-term serial culturing to assess population doubling levels as described in detail by Wang and Gundersen (18) and shown in Table I. In addition, the proliferating potential was measured by thymidine incorporation for assay of the activity of DNA synthesis, as described by Hollenberg and Cuatrecasas (19). In brief, cultures were labeled with 5 ml growth medium containing 1 μCi/ml of (methyl-^{3}H) thymidine. The labeling was carried out for 16 h and the reaction was stopped by rinsing with cold phosphate buffered saline (4°C). The cells were treated with 5% trichloroacetic acid and extracted with methanol. The cell monolayer was hydrolyzed at 70°C for 15 min in 2 ml 0.2 N NaOH. After neutralization with 2 ml of 0.2 N HCl, 1 ml aliquot of the labeled cell material was added to 10 ml scintillation fluid and counted.

RESULTS

Results of immunofluorescence microscopy show that S-30 antigen is localized in the nucleus of in vitro aged fibroblasts derived from both young and old donors (Fig. 1). In cell cultures derived from a donor of 8 fetal wk, the absence of S-30 related nuclear staining was observed in ~98% of the fibroblast population at the early stage of in vitro life span, with CPDL < 10 (Table I and Fig. 1, *a* and *b*). In contrast, the distinct nuclear staining of S-30 antibody was found in 98% of the cells of the same cell strain at a late stage of their in vitro life span, with CPDL > 65 (Fig. 1, *c* and *d*). Similar

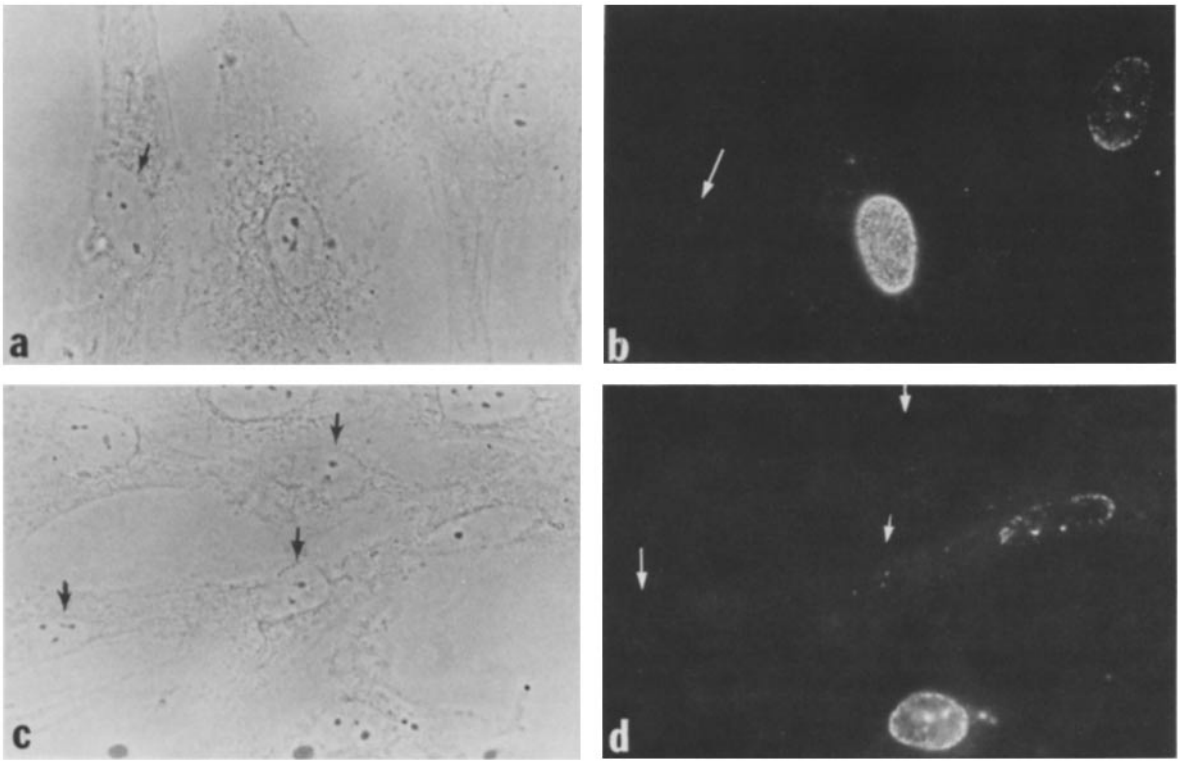

FIGURE 2 The heterogeneous expression of a senescence-specific protein (SSP), S-30, in cultures at the intermediate stage of their life span by phase-contrast (*a* and *c*) and immunofluorescence (*b* and *d*) microscopy. (*a* and *b*) Staining pattern of S-30 in the 0011 cell strain at CPDL = 40. Approximately 30–40% of the cell population shows S-30 staining activity, whereas the remaining subfraction of the cell culture is still negative (arrows). (*c* and *d*) Staining pattern of S-30 in the 3529 cell strain at CPDL = 15. Approximately 20–30% of the cell population shows the positive reaction with S-30 antibody, whereas the remain subfraction is negative (arrows). × 600.

TABLE I

The Presence of S-30 Staining Pattern in Five Selected Strains of Fibroblasts

Repository number	Donor		Earliest available PDLs	CPDL		PDLs to show S-30 staining pattern		
	Age	Sex		Mean	Range	(−)*	(+/−)‡	(+)§
GM0011	8 fetal wk	M	6	65	57–72	6–15	15–55	>65
GM2936B	20 d	M	16	57	54–60	—	16–53	>53
GM0038A	9 yr	F	21	56	52–61	—	21–52	>52
GM2912A	26 yr	M	5	31	27–38	5–10	10–30	>30
GM3529	66 yr	M	3	28	24–32	3–10	10–27	>27

PDLs, population doubling levels.

* Approximately 80–95% of cell population shows negative staining reaction for S-30 antibody.

‡ The percentage of cell population showing positive reaction for S-30 antigens varies in different CPDLs.

§ Approximately 90–95% of cell population shows positive reaction for S-30 antibody.

TABLE II

Comparison between S-30 Staining and Activity of DNA Synthesis

Repository number	0011			3529		
	CPDLs	cpm	S-30 staining	CPDLs	cpm	S-30 staining
Young cells	<10	16,894 ± 250	−	<10	15,951 ± 360	−
Senescent cells	>65	4,206 ± 150	+	>30	3,052 ± 105	+

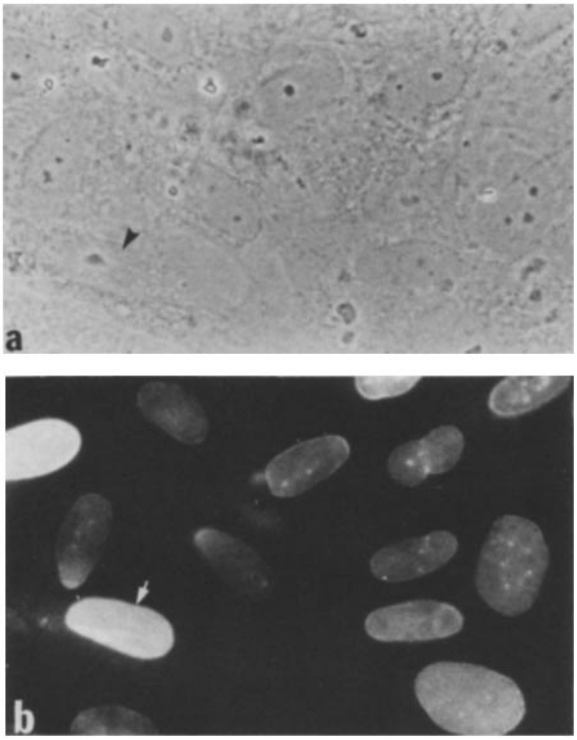

FIGURE 3 S-30 expression in young fibroblasts of confluent cultures. Cultures of 0011 at CPDL < 10 were allowed to grow to confluence and remain at this high cell density for a week before processing for immunofluorescence microscopy with S-30 antibody. (*a* and *b*) Phase and fluorescence microscopy of S-30 staining pattern. In some cells (arrow), the expression of S-30 is quite prominent. × 600.

absence of S-30 staining in most young fibroblasts (CPDL < 10) and its presence in 98% of the senescent fibroblasts (CPDL > 30) was observed in the 3529 cell strain, derived from a 66-yr-old donor (Fig. 1, *e–h*).

S-30 staining was examined more closely with various cultures of human fibroblasts at the intermediate stages of their in vitro replicative life span. Fig. 2 demonstrates that the presence of S-30 in these cultures is heterogeneous, ranging from abundant, as evidenced by brightly stained nuclei, to scarce, as evidenced by the lightly stained nuclear envelope, to absence of staining in ~60% of the cells in cultures. The faint staining of S-30 at the region of the nuclear envelope may indicate that S-30, when it is expressed initially, is located at the outskirts of nuclei, but that once it becomes abundant, it extends its expression throughout the nucleoplasm and nucleoli.

The nuclear staining pattern of S-30 antibody was found in the nonproliferating senescent fibroblasts of all five cell strains (Table I). In contrast, the absence of S-30 staining was found in cultures of their young counterparts, whereas the heterogeneous presence of nuclear staining was observed in the intermediate period of their life span.

The growth state of various cultures used for the immunofluorescence studies as shown in Fig. 1 was assessed by the incorporation of [^{3}H]thymidine. Thymidine incorporation was measured as described in Materials and Methods. Parallel cultures of those cells used in Fig. 1 were quantitated for the activity of DNA synthesis by 16 h of incorporation of [^{3}H]thymidine. Monolayer cultures of both young and senescent cells were harvested at the similar sparse density of 3,550 cells/cm^2. The mean ± SD values of three determinations are expressed as counts per minute of cell-bound radioactivity (Table II).

As illustrated here, cultures of 0011 cell strain showing the predominant presence of S-30 staining have only one-quarter of the level of activity of DNA synthesis of those showing the absence of staining activity. A similar reverse relationship between the activity of DNA synthesis and the presence of S-30 staining was also observed in cell strain 3529, derived from the 66-yr-old donor.

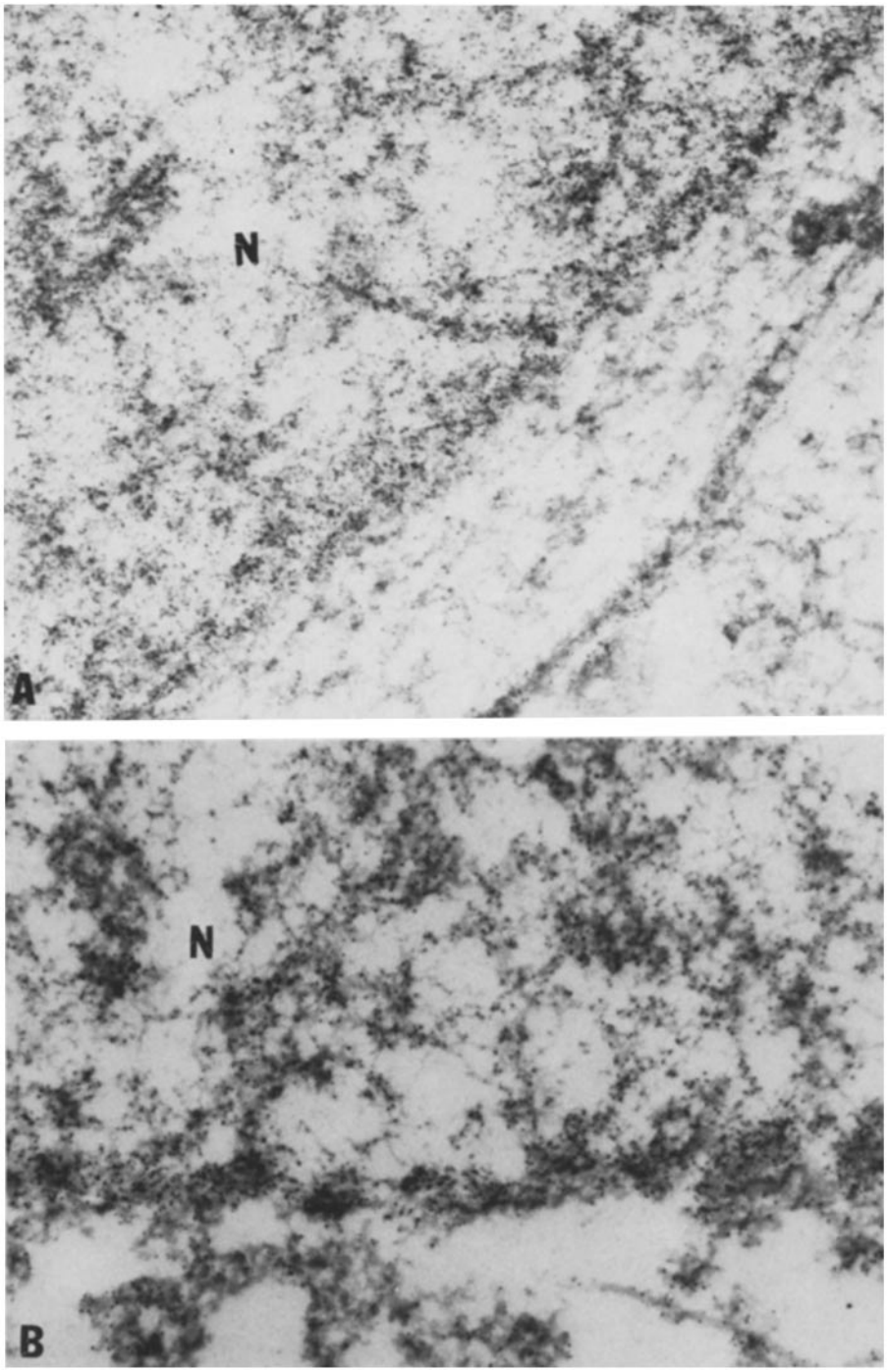

FIGURE 4 Immunogold localization of S-30 antigen in senescent fibroblasts (0011 at CPDL > 65). The antigen-antibody reaction in situ was visualized by further incubation with rabbit antimouse IgG followed by protein-A conjugated with 5-nm gold particles. As illustrated in (*A*), most of the gold particles are located in the nucleus (*N*), with a significant number of gold particles concentrated at the nuclear envelope region. (*A*) × 80,000; (*B*) × 150,000.

The expression of S-30 can be induced in fibroblasts at the early stage of their in vitro life span by arrest of proliferation, such as by contact inhibition of growth and serum starvation. For example, the bright staining pattern of S-30 antibody was observed in the nuclei of young fibroblasts (CPDL = 10; 0011 cell strain) grown to a density of 35,500 cells/cm^2 (Fig. 3). As examined by time-lapse cinematography, the mitotic index at this culturing density is ~2%. The S-30 staining remained in these cells after trypsinization and replating of the confluent cultures. The absence of S-30 was not observed until after the first mitosis in the replated cultures.

The specific cellular location of S-30 antigens was identified by indirect immunoelectron microscopy. S-30 antigens were concentrated in the nucleus as shown by immunofluorescence microscopy (Fig. 4). In some instance, the S-30 staining pattern was observed as patches of clustered gold particles at the nuclear envelope region, with strands of gold decorated strings extending into the interior of the nucleus (Fig. 4).

Biochemical identification of the antigens reacting with S-30 antibody was performed with immunoprecipitation of [^{3}H]leucine labeled cell materials. As shown in Fig. 5, a distinct band located at ~57,000 mol wt was observed with the precipitable materials from senescent cultures of 0011 fibroblasts (CPDL = 60; lanes *8* and *9*) and in confluent

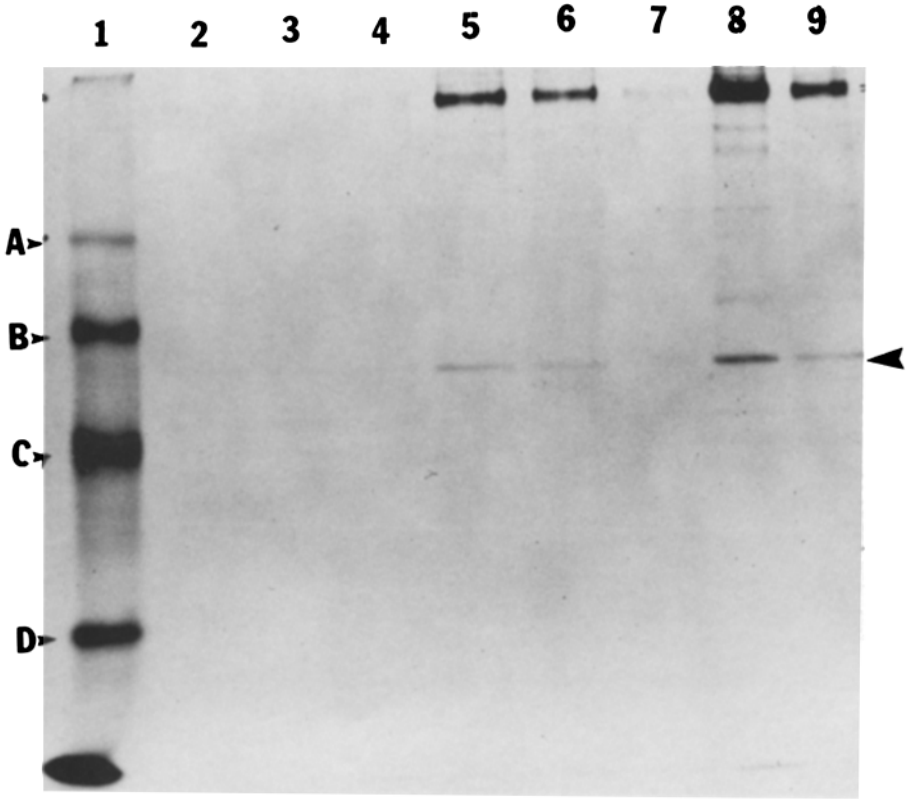

FIGURE 5 Immunoprecipitation with the S-30 monoclonal antibody. The detail is included in Materials and Methods. Whole cell homogenates derived from fibroblasts at different stages of their in vitro life span were used as the substrate for the immunoprecipitation assays. Lane *1*, Molecular weight standards; band A (94,000); band B (68,000); band C (43,000). Lanes *2–3* show absence of any obvious precipitable bands when the 0011 fibroblasts at CPDL = 10 with a cell density of 3,550 cells/cm^2 were used as the substrate. Lane *4*, specimens from the same cell culture were reacted with control medium in place of the hybridoma fluid. Lanes *5–6* show the presence of a protein band at 57,000 mol wt (arrow) as well as the high molecular weight band (double arrow) when the 0011 fibroblasts at CPDL = 10 were grown to high cell density at 35,500 cells/cm^2. Lanes *8–9* show the similar pattern of precipitable bands at 57,000 mol wt and high molecular weight region when the senescent 0011 fibroblasts (CPDL = 60) were used as the substrate. Three times more substrate was used for the immunoprecipitation assays in lanes *5* and *8* than those in lanes 6 and 9. Lane *7* shows the absence of precipitable bands when the hybridoma medium was used in place of the primary hybridoma fluids.

cultures of young 0011 fibroblasts (CPDL = 10; cell density = 35,500 cells/cm^2; lanes *5* and *6*). Trace amounts of the 57,000-mol-wt protein also were observed in cultures at the early stage of 0011 fibroblast life span (CPDL = 10; lanes *2* and *3*). Control experiments showed an absence of S-30 related antigens when the initial incubation was replaced with the hybridoma growth medium (lanes *4* and *7*). In all lanes showing the precipitable bands of 57,000 mol wt, several bands at higher molecular weight were also observed, indicating the possibility of a precursor for this S-30 related protein or contaminants during the process of immunoprecipitation. Pulse-chase experiments are being performed to verify the existence of the precursors for S-30 related antigens.

DISCUSSION

I have demonstrated a protein of ~57,000 mol wt (p57) to be present in nonproliferating fibroblasts that have been aged by in vitro serial passage but absent in their rapidly growing young counterparts. The protein is located in the nucleus of these nonreplicating aged cells. However, the expression of this 57,000-mol-wt protein, recognized by the monoclonal antibody S-30, can be induced in young fibroblasts by means of several culturing techniques that arrest cell growth, such as contact inhibition of proliferation in dense monolayer cultures. In a broader sense, the presence of p57 polypeptide can be used to differentiate nonreplicating cells from their replicating counterparts. Since senescent fibroblasts are defined conventionally by the advent of cessation of replication, S-30 antibody can therefore be used as a marker for in vitro cellular aging.

From the initial experiments with immunogold labeling, I cannot speculate whether the p57 polypeptides are localized exclusively in the matrix component of the nucleus. The harsh treatment of extraction by detergent during the preparative steps for either immunofluorescence or immunoelectron microscopy limits our localization of the S-30 related antigens to the insoluble scaffold. Therefore, my conclusion so far suggests that p57 becomes associated specifically with the nonextractable matrix of the nucleus. Results of future biochemical experiments will reveal to us whether the protein is also present in the soluble fraction of the nucleus in senescent cells.

Preliminary experiments with human skin biopsy have revealed the presence of S-30 related antigens in the nuclei of nondividing terminally differentiated keratinocytes but not in dividing cells located at the basal layer close to the dermis (data not shown). These results demonstrate that markers for senescence need not be restricted to studies of fibroblast aging; they are also useful in studies of other differentiation processes of many types of stem cells, when a common denominator, cessation of replication, acts as the key signal for advancement into the terminal stage of development. The ready applicability of such markers in neoplasm is obvious when their absence, associated with transformation-related cell growth, occurs in tissues destined physiologically to be nonproliferative.

The author expresses her deepest gratitude to Mr. Phil Huie for his excellent technical assistance and Mr. Alan N. Bloch for encouragement and proofreading of this manuscript.

The work was support by funding from research grant AG-3020 from the National Institute on Aging.

Received for publication 5 July 1984, and in revised form 16 October 1984.

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