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Specific Growth Inhibitory Sequences in Genomic DNA from Quiescent Human Embryo Fibroblasts

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We used HeLa cells as recipients in a gene transfer assay to characterize DNA sequences that negatively regulate mammalian cell growth. In this assay, genomic DNA from quiescent human embryo fibroblasts was more inhibitory for HeLa replication than was DNA from either *Escherichia coli* or HeLa cells. Surprisingly, growth inhibitory activity depended on the growth state of the cells from which genomic DNA was prepared; it was strongest in DNA prepared from serum-deprived, quiescent embryo fibroblasts. This latter observation implies a role for DNA modification(s) in regulating the activity of the inhibitory sequences detected in our assay. The level of the observed growth inhibitory activity was sometimes high, suggesting that the relevant sequences may be abundantly represented in the mammalian genome. We speculate that these findings may provide new insights into the molecular mechanisms involved in cellular quiescence and in vitro senescence.

Control of mammalian cell growth appears to be mediated by a balance between positive and negative stimuli. Members of the proto-oncogene family have been intensively studied as effectors of positive growth regulation (3). The molecular mechanisms mediating negative regulation of cell proliferation are, in comparison, less clearly defined. Genes have been cloned for several proteins possessing growth inhibitory activity, including transforming growth factor β, which is bifunctional with respect to replication (8, 29, 44), and members of the interferon family (42); it seems likely, however, that there remains to be identified a much larger number of genes coding for growth inhibitory proteins. Important evidence for negative growth regulation has been obtained from studies of heterokaryons formed by fusion of normal replicating cells with either deeply quiescent cells or senescent cells (6, 20, 23, 26, 39, 40, 45). In such experiments, entry into the S phase of the cell cycle can be strongly inhibited by the nonreplicating partner. Additional evidence for negative growth control has been provided by experiments with somatic cell hybrids for which limitation of proliferative potential (5, 24, 25) and suppression of tumorigenicity (2, 7, 12, 15, 31, 32, 36–38) have been demonstrated by a number of laboratories. Although the heterokaryon and somatic cell hybrid approaches have provided very important insights, they do not afford a straightforward means of obtaining molecular clones of the relevant genes.

We have described the use of a DNA-mediated gene transfer assay to detect genes that can negatively regulate cell growth (R. Padmanabhan, T. Howard, and B. H. Howard, *in* N. Colburn, H. Moses, and E. Stanbridge, ed., *Growth Factors, Tumor Promoters, and Cancer Genes*, in press). In this assay, transfected, reversibly growth-arrested HeLa indicator cells are scored by a bromodeoxy-uridine (BrdUrd)–G-418 resistance double selection. Earlier results indicated that genomic DNA from WI38 human embryo fibroblasts is substantially more inhibitory for HeLa growth than are DNAs from *Escherichia coli* or replicating HeLa cells. We report here that the growth inhibitory activity in human embryo fibroblast genomic DNA can be markedly enhanced when the DNA is harvested from cells rendered quiescent by prolonged serum deprivation. This suggests that growth inhibitory sequence activity may be controlled by DNA modification(s), e.g., by rearrangement, amplification, or methylation events. The potential relevance of these findings with respect to cellular quiescence, terminal differentiation, and in vitro senescence is discussed below.

MATERIALS AND METHODS

Materials. BrdUrd and Hoechst 33258 were purchased from Sigma Chemical Co., St. Louis, Mo.; the antibiotic G-418 was purchased from GIBCO Laboratories, Grand Island, N.Y. Minimal essential Spinner medium without glutamine but with bicarbonate was obtained from HEM Research, Inc.

Preparation of DNA. Genomic DNA was prepared by proteinase K digestion (in 50 mM Tris hydrochloride [pH 7.9]–50 mM EDTA–0.5% sodium dodecyl sulfate for *E. coli* or in 0.5 M EDTA [pH 8.2]–0.5% sarcosyl for mammalian cells [4]) followed by two phenol extractions, extensive dialysis in 10 mM Tris hydrochloride (pH 7.9)–5 mM EDTA–10 mM NaCl, two cycles of cesium chloride-ethidium bromide equilibrium centrifugation, isobutanol extraction, and dialysis into HE (10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid], 1 mM EDTA [pH 7.1]). Bacterial and cellular genomic DNAs were diluted in HE to a concentration of 15 μg/ml and then disaggregated and sheared slightly by passage through a 25-gauge needle at about 0.2 ml/s just before transfection.

Cell culture. HeLa S3 and WI38 cells were obtained from the American Type Culture Collection, Rockville, Md. MRC-5 cells were purchased from Advanced Biotechnologies, Inc., Silver Spring, Md. These cell lines were maintained as monolayer cultures in Dulbecco modified Eagle medium supplemented with penicillin (50 U/ml), streptomycin (50 μg/ml), glutamine (2 mM), and 10% fetal bovine serum. HeLa cell Spinner medium was supplemented with 10% filtered horse serum and 2% fetal bovine serum. WI38, HeLa, and WI38-transfected HeLa cells were checked after culture in our laboratory to exclude mycoplasma contamination.

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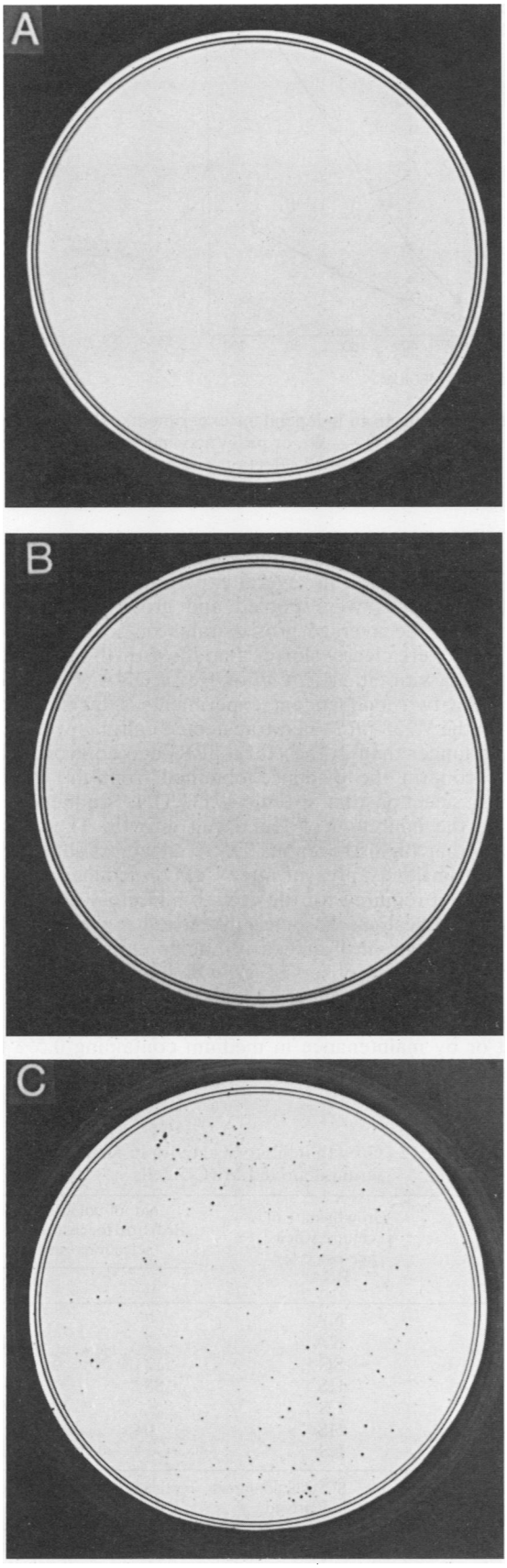

DNA-mediated transfection. HeLa S3 cells were plated at 7.5×10^5 cells per 75-cm^2 flask and refed the next day at 3 to 4 h before transfection. Calcium phosphate-DNA coprecipitates contained 5 μg of pRSVneo (10) and 15 μg of human fibroblast, HeLa S3, or *E. coli* DNA. At 4 h after transfection, cells were washed with Dulbecco modified Eagle medium, treated with 15% (wt/vol) glycerol in HEPES-bufferedsaline for 30 s, washed with Dulbecco modified Eagle medium, and refed. A 48-h period was allowed for phenotypic expression of exogenous DNA, after which the cells were trypsinized, counted, and placed in Spinner medium at a density of 2.5×10^5 to 3×10^5 cells per ml. Relative pRSVneo transfection efficiencies were checked by removing about 10^5 cells from the Spinner culture and plating this sample at a density of 200 cells per cm^2 in G-418 medium (standard monolayer medium supplemented with 800 to 1,000 μg of G-418 per ml). BrdUrd and freshly prepared Hoechst 33258 were then added (final concentrations, 100 and 1 μg/ml, respectively) into foil-wrapped Spinner cultures, and the incubation was continued for an additional 44 h. Cells were exposed for 5 min to fluorescent light (25 W/m^2 from two Sylvania F15T fluorescent bulbs positioned about 4 cm from the Spinner bottle), counted, and plated at 10^4 cells per cm^2 in G-418 medium. In some experiments, Hoechst 33258 was not added until 4 h before exposure of the cells to fluorescent light. Selection for the pRSVneo marker was maintained by refeeding with G-418 medium at 4-day intervals.

Growth curves. G-418-resistant colonies maintained in selection medium for at least 16 days were pooled, replated into replicate T-25 flasks with G-418 medium, and counted at approximately 24-h intervals. Doubling times were calculated from cell counts taken at 24 and 72 h after plating.

RESULTS

Assay for growth inhibitory activity. Genomic DNAs were prepared from quiescent cultures of WI38 human embryo fibroblasts in which quiescence had been induced either by density arrest in medium containing 10% fetal calf serum or by serum deprivation in medium containing 0.5% fetal calf serum. The latter (low-serum) condition has previously been shown to induce a characteristic deep G_0 state of quiescence that mediates growth inhibition in heterokaryon experiments (1, 6, 26, 30, 39). These DNAs were mixed with the marker plasmid pRSVneo (10) and transfected into HeLa S3 cells growing in monolayer culture. As controls, parallel transfections were done in which either *E. coli* DNA or genomic HeLa DNA prepared from a subconfluent replicating culture was substituted for WI38 DNA. At 48 h after transfection, cells were placed in Spinner culture, and a BrdUrd-Hoechst 33258 selection against replicating cells was done (41). Finally, cells surviving the BrdUrd-Hoechst selection were plated in G-418 medium to select for pRSVneo expression (35).

FIG. 1. BrdUrd–G-418 selection for DNA-mediated growth inhibition. HeLa cells were transfected with pRSVneo and *E. coli* DNA (A), pRSVneo and HeLa genomic DNA (B), or pRSVneo and WI38 genomic DNA (C). HeLa DNA was prepared from a subconfluent replicating culture; WI38 DNA was from a serum-deprived quiescent culture. Colonies shown were obtained after BrdUrd-Hoechst treatment to eliminate transiently nonreplicating cells and after selection of surviving cells in G-418 medium for about 20 days. Colony counts and associated pRSVneo transfection efficiencies are presented in Table 1.

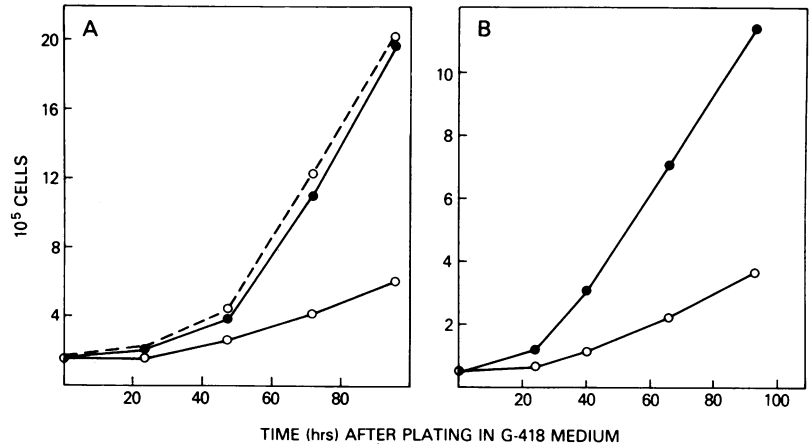

FIG. 2. Growth curves of BrdUrd–G-418-selected cultures. Panels A and B show data from independent experiments. HeLa cells were transfected with pRSVneo and *E. coli* DNA (O– – –O), pRSVneo and HeLa genomic DNA (●——●), or pRSVneo and WI38 genomic DNA (O——O) and then subjected to the BrdUrd–G-418 double selection. After at least 16 days in G-418 selection medium, colonies from each of the cultures were pooled and replated to determine average growth rate.

In this experimental protocol, the number of G-418-resistant colonies obtained should provide an approximate measure of the number of transfected cells that did not replicate during the BrdUrd-Hoechst selection step. Since variables that might nonspecifically inhibit HeLa replication (e.g., exposure to calcium phosphate-DNA precipitates or BrdUrd) were identical in each transfection, the results should indicate whether DNAs from different cell types exhibit characteristically different growth inhibitory activities. Data from one such experiment are shown in Fig. 1 and Table 1. No colonies formed in mock- or *E. coli*-pRSVneo-transfected cultures, whereas a small number of colonies remained after control transfection with the HeLa-pRSVneo mixture. By comparison, transfection with pRSVneo and either of the WI38 DNA preparations yielded at least 20-fold more colonies than the controls, indicating a surprisingly high level of growth inhibitory activity in those DNAs. The large differences between these preparations and those of *E. coli* and HeLa control DNAs indicated that the major component of the WI38 growth inhibitory activity was specific. Interestingly, WI38 DNA from the serum-deprived culture gave two times as many colonies as DNA from the high-serum, density-arrested culture. This latter difference, although small, suggested the possibility that relative inhibitory levels in WI38 DNA preparations reflect the growth states of the cells from which they were harvested (see below).

Growth of selected cell populations. Average replication rates of cells after transfection with the various DNA mixtures were compared in several experiments. When G-418-resistant colonies were pooled and growth curves were determined, the average growth rates of cells transfected with WI38 were clearly slower than the growth rates of cells transfected with either *E. coli* or HeLa DNA (Fig. 2A and B). In the two independent experiments shown, doubling times in the WI38-pRSVneo-transfected cultures were 1.4 to 1.8-fold longer than in the HeLa-pRSVneo controls. These results confirm the evidence obtained from the BrdUrd-Hoechst selection that specific WI38 DNA sequences can mediate the inhibition of HeLa cell growth. They further indicate that high exogenous DNA concentrations, which may be transiently present after $CaPO_4$-mediated transfection, are not required for the growth inhibitory effect.

DNA modification. To determine whether genomic DNA from another strain of quiescent human embryo fibroblasts could mediate the observed growth inhibitory effect, we prepared DNA from quiescent MRC-5 human embryo fibroblasts. As before, quiescence was induced either by density arrest or by maintenance in medium containing 0.5% fetal calf serum. Results of experiments comparing DNAs from

TABLE 1. Comparison of growth inhibitory activities and frequencies of G-418-resistant colonies

Donor DNA	Growth state of culture when harvested for DNA[a]	Frequency of stable transfection (pRSVneo marker)	No. of colonies after BrdUrd-Hoechst and G-418 selections
None	NA	0	0
E. coli	NA	3×10^{-3}	0
HeLa S3	SC	1×10^{-2}	3
WI38	HS	1×10^{-2}	70
WI38	LS	1×10^{-2}	148

[a] NA, Not applicable; SC, subconfluent, replicating; HS, high serum, density arrested; LS, serum deprived.

TABLE 2. Growth inhibitory activity in DNA from serum-deprived MRC-5 cells

Donor DNA	Growth state of culture when harvested for DNA[a]	No. of colonies after BrdUrd-Hoechst and G-418 selections[b] in expt:	
		1	2
None	NA	7	0
E. coli	NA	7	18
HeLa S3	SC	12	2
WI38	HS	88	
WI38	LS		450
MRC-5	HS	0	8
MRC-5	LS		190

[a] NA, Not applicable; SC, subconfluent, replicating; HS, high serum, density arrested; LS, serum deprived.

[b] Note that absolute numbers of colonies in a given experiment vary as a function of multiple variables, including transformation efficiency, serum and G-418 lots, etc.; accordingly, comparisons between numbers of colonies are most valid within a single experiment.

quiescent WI38 and MRC-5 fibroblasts are shown in Table 2. High levels of inhibitory activity in MRC-5 DNA were observed only when MRC-5 cells had been forced into quiescence by prolonged serum deprivation. In additional repeat experiments with independent DNA preparations, MRC-5 DNA from replicating (subconfluent) or density-arrested, high-serum cultures consistently failed to exhibit growth inhibitory activity, whereas MRC-5 DNA prepared from serum-deprived cultures was strongly inhibitory (data not shown). An important and rather unexpected implication of these data is that DNA modification(s) (e.g., rearrangement, amplification, methylation, or a more novel mechanism) appears to modulate the activity of growth inhibitory sequences detected in this assay.

DISCUSSION

Our interest in negative regulation of mammalian cell growth led us to explore the characteristics of a gene transfer assay in which HeLa cell replication is inhibited by the introduction of genomic DNA from human embryo fibroblasts. The results reported here indicate that the activity of the donor DNA in this assay reflected the growth state of the cells from which that DNA was prepared and was highest in embryo fibroblasts rendered quiescent by prolonged serum deprivation.

The enhancement of growth inhibitory activity in our assay by a deep G_0 state of quiescence appears to parallel the results obtained in heterokaryon experiments (1, 6, 26, 30, 39), suggesting that growth inhibition may be mediated by similar mechanisms in both assays. On the other hand, it has been reported that HeLa cells exhibit a dominant phenotype in heterokaryon fusions, i.e., induce DNA replication in nuclei from quiescent or senescent cells (19, 23, 26, 39). One exception was a report by Norwood et al. (19), who, although arguing that the HeLa phenotype is dominant, also presented evidence that HeLa replication can be partially inhibited at later times (48 to 72 h) after fusion with senescent cells. Several differences between the heterokaryon assay and our assay may account for the apparently discrepant results obtained. First, our assay scores inhibition of replication only, whereas the heterokaryon experiment measures a net difference between potentially simultaneous inhibition and stimulation. Second, growth inhibitory sequences introduced as purified DNA in conjunction with the pRSVneo expression plasmid may be more resistant to repression by *trans*-acting HeLa factors (or more prone to irreversible activation) than are the same sequences in the heterokaryon protocol. Third, the use of Spinner culture in the BrdUrd-Hoechst selection step in our assay may sensitize HeLa cells to the growth inhibitory mechanism(s). HeLa S3 cells have been shown to be more susceptible to serum factor-dependent G_1-G_0 arrest when maintained in suspension culture than when maintained in monolayer culture (22). In heterokaryon experiments, the fused cells are maintained in monolayer conditions during the thymidine labeling interval; thus, the inhibitory mechanism(s) may be relatively ineffective. This last point is in agreement with our experience that specific WI38 growth inhibitory activity is considerably more difficult to detect if the BrdUrd-Hoechst selection is carried out in monolayer culture (unpublished results).

Two explanations may be offered for the higher growth inhibitory activity in WI38 DNA than in MRC-5 DNA when both DNAs were prepared from high-serum, density-arrested cultures. First, this disparity may reflect cell strain differences (14). It is possible that WI38 cells, but not MRC-5 cells, approach a true G_0 state after being maintained at confluence for 4 to 8 days in high-serum conditions. That WI38 cells can enter a still deeper state of quiescence, however, is suggested by a small additional increase in growth inhibitory sequence activity observed when WI38 DNA is prepared from a serum-starved culture (Table 1). A second potential explanation for the difference between WI38 and MRC-5 cells relates to mean population doubling levels. Although the passage level of the WI38 cells that we used as the source of donor DNA was not precisely defined, these cells showed (in comparison to MRC-5 cells) several characteristics of early senescence, including increased doubling times and about a 50% decrease in density at confluence (unpublished results). Conceivably, growth inhibitory sequences are gradually activated as human embryo fibroblasts approach and enter in vitro senescence. These two explanations are not mutually exclusive; for example, it is possible that as such cells reach late passage levels, they more readily enter a state of deep quiescence. If in vitro senescence turns out to be an independent variable in our results, it would represent a second parallel to the heterokaryon assay (6, 20, 23, 26, 40, 45).

One interesting speculation that follows from the idea that growth inhibitory sequences can become activated is that such activation might play a role in the cessation of cell replication associated with terminal differentiation or in cellular senescence. Particularly if the sequences detected in our assay are present in many copies (as the surprisingly strong inhibitory activity in serum-deprived, quiescent fibroblasts suggests), then the rate of activation could be quite high. Certain types of activation mechanisms would be expected to exhibit stochastic characteristics, which in fact have been observed in differentiation (11, 16, 43) and cellular senescence (13, 34). Unique aspects of the processes of quiescence, senescence, and terminal differentiation could reflect either different modes of activation or activities of different sequences. Alterations in the genome in general and repeated sequences in particular have been proposed to play a role in in vitro senescence (9, 18, 21, 28, 33), but to our knowledge, evidence has not been presented that activation of repeated sequences could mediate growth inhibitory activity.

For a more complete understanding of the results reported in this paper, it will be necessary to obtain molecular clones that possess growth inhibitory activity as shown by our assay. To this end, we constructed cosmid libraries from *E. coli*, HeLa, and WI38 genomic DNAs. These cosmid libraries were found to exhibit activities similar to the genomic DNAs from which they were derived., i.e., the WI38 DNA library yielded 5- to 10-fold more G-418-resistant colonies than either the *E. coli* or HeLa DNA libraries (R. Padmanabhan, M. McCormick, T. H. Howard, M. Fordis, and B. H. Howard, unpublished results).

The finding of differential growth inhibitory activities in the WI38 versus HeLa or *E. coli* cosmid libraries argues against the trivial possibility that growth suppression is mediated by modified DNA in a non-sequence-specific manner. This finding is more consistent with the possibility that DNA rearrangements or amplification involving growth inhibitory sequences contributes to increased activity. DNA modifications could also affect inhibitory activity if such modifications altered the initial cloning efficiency (and hence the eventual representation) of relevant sequences in the various libraries. Sequence-specific single-strand nicks or gaps, e.g., in HeLa genomic DNA, would be expected to impair cloning competence. DNA methylation could also

affect cloning; for example, one aspect of the poison phenotype, i.e., the inefficient reestablishment of pBR322 derivatives in *E. coli* after replication in mammalian cells, has been postulated to reflect changes in methylation of specific sequences in such plasmids (17). Recently, a 5-methylcytosine-dependent restriction system was reported to exist in several *E. coli* strains (27), including HB101, the strain used in our studies.

The speculation that the strong growth inhibitory signal exhibited by the WI38 and quiescent MRC-5 DNAs may reflect an unexpected abundance of sequences with the potential to suppress growth is further supported by experiments in which randomly selected WI38 cosmid sublibraries, with complexities of less than 1,000 clones, scored positive in our assay (M. McCormick, R. Padmanabhan, T. H. Howard, M. Fordis, and B. H. Howard, unpublished data). Such experiments cannot distinguish between activity mediated by multiple different, unrelated sequences versus that mediated by one or a small number of repetitive sequence elements, although from the standpoint of simplicity, we favor the latter alternative. In either event, our results strongly suggest that it will be possible to isolate these sequences and to define their role(s) in the negative regulation of mammalian cell growth.

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