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HUTCHINSON-GILFORD PROGERIA FIBROBLASTS EXHIBIT METABOLICALLY NORMAL URIDINE UPTAKE AND RNA SYNTHETIC RATES

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The accelerated aging disease Hutchinson-Gilford progeria syndrome displays altered messenger RNA levels in cultured fibroblasts, yet little is known of effects on transcription by RNA polymerases other than RNA polymerase II. Total RNA metabolism was examined by incubation of Hutchinson-Gilford progeria fibroblasts with [5-^{3}H]uridine in asynchronous culture. Uptake of radiolabel was quantitative and was incorporated preferentially (99±0.3%) into newly synthesized RNA. Progeria and control cultures showed comparable rates of uptake of radiolabel, time courses of RNA synthesis, and relative intensity profiles of newly synthesized ribosomal RNA. These characteristics held over a greater than ten-fold range of cell densities ($5x10^3$ to $8x10^4$ cells/cm^2). Progeria and control fibroblasts thus have comparable metabolic capacities for uridine uptake and net RNA production, emphasizing the relative specificity of transcriptional changes previously identified in progeria cells.

Hutchinson-Gilford Progeria syndrome (progeria) is a fatal disease of childhood with features resembling normal aging. Children with progeria typically appear normal at birth although scleroderma, midfacial cyanosis and a sculptured nose may be apparent (1). Generally, symptoms appear during the first year, with one or a combination of growth retardation, alopecia or abnormalities of the skin leading to initial presentation of the child and diagnosis of progeria. The effects of progeria are primarily upon connective tissue including skin, with significant cardiovascular effects. The progression of the disease is relatively rapid, culminating in death at a median age of 12 years (2). In over 80% of cases death is attributable to complications arising from atherosclerosis, including congestive heart failure, myocardial infarction and coronary thrombosis (1). There is no cure or effective treatment for progeria. Progeria is considered to be the most appropriate model disease for the study of aging (2, 3).

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Abbreviations: TCA, trichloroacetic acid; PD, population doublings.

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Consequently, basic research into progeria provides not only insights into the disease itself but also offers the potential of a greater understanding of diseases frequently associated with the normal aging process.

Recently considerable interest has developed in the investigation of specific messenger RNA transcript levels in progeria, with reports of abnormalities in the abundance of transcripts for tropoelastin (4, 5), α_1 and α_2 type IV procollagen (5), platelet-derived growth factor A-chain (6), c-fos (6, 7), c-myc (8), and insulin receptor (9). Levels of these apparently disparate transcripts are presumably a consequence of the progeria genotype and contribute to the perception that altered transcript levels predominate in progeria. Yet RNA polymerase II transcripts are a minor component of the total cellular RNA, of which ribosomal RNA is the major species. One phenotypic consequence that is proposed on the basis of these data is a broad effect on RNA metabolism. To exclude contributions from such a broad effect we demonstrate here that total transcriptional activity is remarkably similar in progeria and control fibroblasts.

Methods

Routine Cell Culture

Cell strains GM02037A and AG03513B (Coriell Cell Repositories, NJ, USA), established from clinically normal and progeria donors respectively, were used in these studies, and were cultivated *in vitro* as previously described [10]. Cells were monitored with respect to growth behaviour and morphology. Strain AG03513B morphology was as described for this progeria strain and cells consistently displayed *in vitro* growth characteristics typical of cultured progeria fibroblasts [10]. The average growth rate was 0.2 PD per day (GM02037A was 0.4 per day) and the maximal *in vitro* lifespan was 41 PD (GM02037A was 70PD).

Distribution of TCA-Insoluble Radioactivity between DNA and RNA

The radioactive nucleoside was [5-^{3}H]uridine (Amersham International plc, Buckinghamshire, UK) and was diluted to 1 μM uridine (5 μCi/ml) in Eagle's minimum essential medium with Earle's salts, non-essential amino acids and glutamine and containing 10% fetal calf serum. Cultures were incubated for 2 h, after which time the labelling medium was removed, and the culture rinsed with phosphate buffered saline. Cells were harvested by trypsinization, and centrifuged (2000 rpm, 5 min, 4°C). To each cell pellet was added 2.0 ml lysis buffer (0.6% w/v SDS in 10 mM Tris-Cl, 1 mM EDTA, pH 7.4; [11]), and the sample vortexed for 15 s at room temperature.

Cell lysate aliquots were used for determination of the total incorporation of radioactivity by precipitation in trichloroacetic acid (TCA). The remainder of each of the cell lysates was used for isolation of total nucleic acid [12]; one aliquot of which was subjected to TCA precipitation, and a second aliquot was incubated with DNase-free RNase A (10 μg/ml; Boehringer Mannheim) prior to TCA precipitation.

Results and Discussion

Development and Efficacy of Assay

The use of [5-^{3}H]uridine as a specific label of RNA synthesis was first proposed by Hayhoe and Quaglino [13]. Adams cast doubt on the suitability of this

method when he reported that up to one-third of the acid-insoluble radioactivity was present as DNA [14]. Although this method has since been used to investigate RNA synthesis in fibroblasts *in vitro* (see, for example, [11]), the question of the relative proportion of incorporated label in RNA and DNA has not been satisfactorily addressed. We addressed the specificity of the labelling protocol under our conditions, to examine the reliability of using the incorporation of [5-^{3}H]uridine into TCA insoluble material for analysis of net transcriptional activity. After incubation of cultures in labelling medium isolated total nucleic acid samples were incubated with RNase A to permit estimation of the proportion of incorporated label present in material other than RNA. The RNase A-resistant portion of the extracted nucleic acids was 1.0±0.3% of the TCA insoluble material in both progeria- and control-derived samples, demonstrating that incorporation into RNA accounts for 99±0.3% of the TCA insoluble radioactivity in the cell lysates under these conditions. The procedure is accordingly suitable for investigation of aspects of RNA synthesis in skin fibroblasts *in vitro*.

Labelling of Progeria and Control Fibroblasts

Incorporation of radioactivity in purified total RNA was similar in progeria and control cell strains, the RNA yields being 2.33±0.30 pmol [5-^{3}H]uridine/10^5 cells and 2.45±0.30 pmol [5-^{3}H]uridine/10^5 cells, respectively. Upon electrophoresis no quantitative differences were seen between progeria and control total (principally ribosomal) RNA profiles, visualized through ethidium bromide staining, or in newly synthesized ribosomal RNA, visualized by fluorography (results not shown). These data are consistent with previous reports that the rate of rRNA processing is not altered as a result of *in vitro* aging of human fibroblast cultures [15, 16], although this is the first demonstration of such an effect in progeria.

Uptake of exogenous uridine, at the concentration used in the present experiments, occurs by facilitated diffusion [17, 18]. The uptake of [5-^{3}H]uridine by progeria and control cells was examined by incubation in labelling medium of duplicate cultures of each cell strain for 2, 5, and 10 min. The average cell density was 2.4±0.2x10^4 cells/cm^2 and 3.4±0.5x10^4 cells/cm^2 in the progeria and control cultures, respectively. For each of the cell lysates the total amount of radioactivity present (i.e., free and incorporated label) was determined. The average rate of uptake in progeria cultures was 379±74 fmol [5-^{3}H]uridine/10^5 cells/min and in the control cultures it was 427±115 fmol [5-^{3}H]uridine/10^5 cells/min. Rates of uptake were comparable by Student's t test ($p < 0.7$). The demonstration of no detectable differences in the uptake of uridine between the two cell strains indicates an absence of significant abnormalities in the functioning of the carrier in progeria fibroblasts.

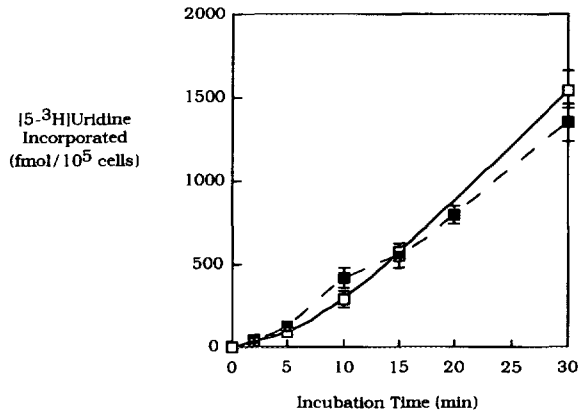

Figure 1. Time course of RNA synthesis by cultured control GM02037A (open symbols) and progeria AG03513B (closed symbols) skin fibroblasts. Each point represents the average of two analyses performed in triplicate for each cell strain and each time point. Error bars indicate standard deviation.

Progeria and control fibroblasts were incubated with labelling medium at average cell densities of $2.4\pm0.2\times10^4$ cells/cm^2 and $3.1\pm0.7\times10^4$ cells/cm^2, respectively. These values lie within the routine range of exponential growth of progeria and control fibroblast cultures (data not shown). Incorporation of radioactivity into TCA insoluble material followed the same course when assayed over a 30 min period (Figure 1). This demonstrates similar levels of RNA synthesis in the control and progeria cell strains at these cell densities.

Cell Density Effects

To exclude contributions due to cell density effects, duplicate flasks for each of progeria and control fibroblasts were seeded at cell densities from 0.5×10^4 cells/cm^2 to 8×10^4 cells/cm^2 and the cells allowed to plate overnight. Characteristic morphology was maintained over the entire range of densities. However the control cultures, which normally show parallel arrays of cells at and approaching confluence, exhibited a greater tendency to disorganized growth patterns at the highest seeding densities. Cultures were incubated with labelling medium for 2 h. A correlation between cell density and incorporation of [5-^{3}H]uridine into TCA insoluble material was seen (Figure 2); for the progeria cultures the correlation coefficient was 0.73 and for the control cultures this was 0.83. Both strains demonstrated comparable dependence of incorporation on cell density: a decrease of 240 fmol [5-^{3}H]uridine per 10^5 cells for every increase of 10^4 cells/cm^2 in cell density (Figure 2). To examine effects at lower density values, duplicate cultures of control and progeria fibroblasts were seeded at cell densities from 0.5×10^4 cells/cm^2 to 3.5×10^4 cells/cm^2, and the cells allowed to plate overnight. Both cell types maintained characteristic morphologies over this

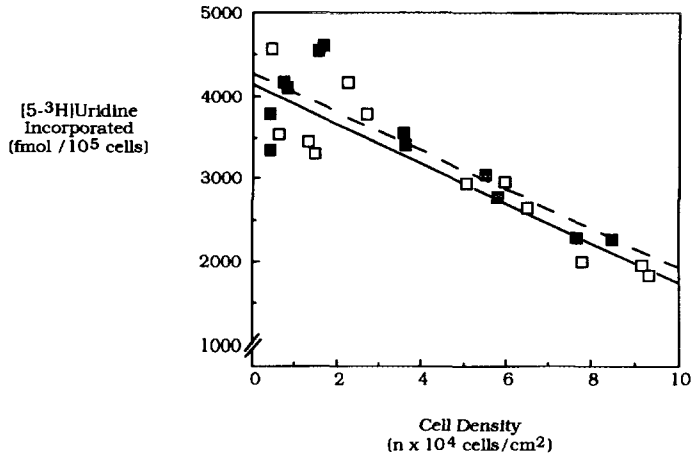

Figure 2. RNA synthesis by cultured control GM02037A (open symbols) and progeria AG03513B (closed symbols) skin fibroblasts as a function of cell density.

range of cell densities. Cultures were incubated with labelling medium for 2 h. Incorporation of label was not substantially affected by cell density up to approximately $3x10^4$ cells/cm^2 (data not shown).

Uptake of [5-^{3}H]uridine from the medium was consistently linear with respect to time when assayed over 10 min. The rates of nucleoside uptake and incorporation of radiolabel into TCA insoluble material were influenced by cell density, with both parameters inhibited by increasing density up to at least $9x10^4$ cells/cm^2. These results are consistent with data using untransformed animal cells *in vitro* (reviewed in [19]). In the present work, when progeria and control cell strains were assayed at similar densities, the magnitudes of uptake and extents of incorporation were statistically equivalent. As the rate of incorporation of label into TCA insoluble material provides an indication of the net rate of RNA synthesis (11), it can be concluded that net RNA synthetic rates in progeria and control fibroblasts are affected by cell density in a similar manner. Indeed progeria fibroblasts appear metabolically unaltered in terms of uridine metabolism leading to and including net RNA production. This is in dramatic contrast to abundance changes observed with a range of mRNA transcripts (4-9) and underscores the specificity of these transcriptional alterations in progeria.

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