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Extensive Homology of Nuclear Ribonucleic Acid and Polysomal Poly(adenylic acid) Messenger Ribonucleic Acid between Normal and Neoplastically Transformed Cells†

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ABSTRACT: A cell line, designated BP6T, derived from Syrian hamster embryo (SHE) cells following treatment with benzo[*a*]pyrene is capable of producing tumors in newborn hamsters following the injection of as few as 1–10 cells. Polysomal poly(A) mRNA and total nuclear RNA obtained from this highly tumorigenic cell line were compared to RNAs obtained from the nonneoplastic parental embryo cells by a variety of techniques. RNA excess hybridizations to normal cell radiolabeled single-copy DNA or to a single-copy DNA tracer enriched for sequences transcribed in neoplastically transformed cells were unable to detect any significant differences in RNA sequence complexity between normal SHE cells and neoplastic BP6T cells. This finding of extensive homology of polysomal poly(A) mRNA and total nuclear RNA between normal and neoplastic cells, together with our previous finding of extensive homology of the major ^{35}S-labeled nuclear or cytoplasmic polypeptides observable on two-dimensional gels [Leavitt, J. C., & Moyzis, R. K. (1978) *J. Biol. Chem. 253*, 2497–2500], demonstrates that the phenotypic changes associated with neoplastic transformation by chemical carcinogens are accompanied by relatively few changes in the qualitative pattern of gene expression in cells cultured in vitro.

Using various nucleic acid hybridization techniques, investigators frequently have reported that changing patterns of RNA transcription and/or processing occur during the differentiation of eucaryotic cells and tissues (Gelderman et al., 1971; Brown & Church, 1972; Grouse et al., 1972; Ryffel & McCarthy, 1975; Galau et al., 1976; Axel et al., 1976; Young et al., 1976; Hastie & Bishop, 1976; Kleene & Humphreys, 1977; Chikaraishi et al., 1978; Caplan & Ordahl, 1978; Wold et al., 1978). These numerous reports of extensive changes in RNA populations accompanying developmental processes have lent support to theories that suggest that differential gene expression is the mechanism underlying developmental change (Davidson, 1976). Similarly, it is widely believed that changes in gene expression must accompany the transformation of cells from a normal to a neoplastic phenotype (Heidelberger, 1975; Mintz & Illmensee, 1975; Nowell, 1976; Stein et al., 1978). Experimental reports of the actual extent of change in RNA expression accompanying neoplastic transformation are not numerous, however. To our knowledge, the only published reports analyzing changes in RNA sequence complexity in transformed cells by the method of saturation of a radiolabeled single-copy DNA tracer with excess RNA were those of Grady & Campbell (1973, 1975). These authors found massive differences in the sequence complexity of nuclear or polysomal RNA isolated from "normal" mouse AL/N cells as compared to nuclear or polysomal RNA isolated from polyoma virus "transformed" cells (PY AL/N). On the basis of these results, the authors postulated that neoplastic transformation, like many other normal developmental changes in cellular phenotype, was also accompanied by extensive changes in gene expression.

Recent studies in other systems, however, using an alternate method to measure RNA sequence complexity (e.g., the reassociation of RNA with its cDNA transcript; Bishop et al., 1974) have failed to confirm this extensive reprogramming of gene expression (Williams et al., 1977; Getz et al., 1977; Rolton et al., 1977). In general, little or no change was observed in the poly(A) RNA populations studied by these investigators. The cDNA–poly(A) RNA reassociation technique used in these more recent studies is considered, however, to be less sensitive than the single-copy DNA saturation technique in determining the total sequence complexity of an RNA population. This lack of sensitivity is attributable both to uncertainties in the exact assignment of kinetic components in heterogeneous reactions and a potential bias for underrepresentation of the most complex class of RNAs in the cDNA transcript. [For example, see Quinlan et al. (1978) and Meyuhas & Perry (1979) or compare Ryffel & McCarthy (1975), Hastie & Bishop (1976), and Young et al. (1976).] It is possible, therefore, that the differences reported may, in part, result from the use of different experimental methods.

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We chose, therefore, to investigate the extent of transcriptional change in neoplastically transformed cells by the method of saturation hybridization of isolated single-copy DNA with excess RNA, as originally used by Grady & Campbell (1973, 1975). The in vitro transformation of Syrian hamster embryo (SHE) cells is used extensively for mechanistic studies of neoplastic transformation and as an assay system for potential carcinogens (Berwald & Sachs, 1963; DiPaolo & Donovan, 1967; Pienta et al., 1977; Barrett & Ts'o, 1978). Using this system, we have selected for a reasonably homogeneous population of neoplastically transformed SHE cells and obtained a cell line (BP6T) capable of producing tumors in newborn hamsters following the injection of as few as 1–10 cells. The results reported here on RNA expression in this cell line demonstrate that neoplastic transformation results in no detectable change in the sequence complexity of polysomal poly(A) mRNA. These results confirm those obtained by the cDNA–poly(A) mRNA technique used by Getz et al. (1977) in their study of mRNA sequence complexity in chemically transformed cells. Additionally, an analysis of the sequence complexity of total nuclear RNA between normal and neoplastically transformed cells suggests extensive homology in this RNA population as well.

Experimental Procedures

Animals, Cell Culture, and Tumorigenicity. Syrian hamsters (*Mesocricetus auratus*) were obtained from Lakeview Hamster Colony, Newfield, NJ (LSH/ssLAK strain), or the Leo Goodwin Institute, Fort Lauderdale, FL (Graffi strain). Syrian hamster embryo cell cultures were established from 12–13-day embryos removed aseptically from inbred Syrian hamsters, strain LSH/ssLAK. Pools of primary cultures from littermates were stored in liquid nitrogen, and secondary cultures were initiated from the frozen stocks. The derivation of the cell lines used in this work is diagrammed in Figure 1 and explained in the text. SHE and SHE 21 C1–2 cell cultures are diploid, while 18 C1–10, BP6, and BP6T cell cultures are subdiploid (Moyzis, 1978). The growth medium used was IBR modified Dulbecco's Eagle's reinforced medium (Biolabs, Northbrook, IL) supplemented with 0.22% (w/v) $NaHCO_3$ and 10% Rehatuin fetal bovine serum (Reheis Chemical Co., Kankakee, IL) without antimicrobial agents. Cells were subcultured by trypsinization with 0.1% trypsin solution (1:250, Grand Island Biological Co., Grand Island, NY) for 5 min at 37 °C. All cultures were checked for the presence of mycoplasma by both a biochemical assay (Levine, 1972) and an agar culture assay (Microbiological Associates, Walkersville, MD) and found to be negative.

Quantitative tumorigenicity was measured as follows. Cells growing in plastic 75- or 150-cm^2 T-flasks (Falcon, Oxnard, CA; Costar, Cambridge, MA) were dissociated with 0.1% trypsin, pelleted at 2500*g* for 10 min, and resuspended in growth medium, with or without fetal bovine serum. Visual microscopic observation was used to ensure that a single cell suspension had been obtained. Cell density was determined in a coulter counter, and serial dilutions of the cells in growth medium were performed down to 10^0 cells/0.1 mL. Cell density was rechecked with a hemocytometer at each dilution. Three-day-old Syrian hamster littermates (strain LVG/LAK, outbred, Lakeview Hamster Colony), neither X-irradiated nor thymectomized, were inoculated subcutaneously on their ventral surface with 0.1 mL of the appropriate cell dilution. The hamsters were weaned and sexed at 21–28 days of age and examined weekly for the development of palpable tumors. All neoplastic cell lines described in this paper grew progressively, forming invasive anaplastic fibrosarcomas, and ultimately killed their hosts. Control hamsters were maintained for 12 months before sacrifice.

Polysome Purification and Isolation of Poly(A) mRNA. All glassware used in the purification of RNA was either acid-washed and rinsed with double-distilled, autoclaved water or baked at 300 °C overnight. All solutions were either autoclaved twice or treated with DEP (diethyl pyrocarbonate; Eastman Organic Chemicals, Rochester, NY). Column chromatography was conducted with autoclaved gels or gels treated with NaOH and DEP.

SHE, SHE 21 C1–2, 18 C1–10, and BP6T cells were grown in 150-cm^2 T-flasks as described above. In midlog growth phase, the flasks were placed in a 0 °C ice bath, the medium was removed, and the cell monolayer was washed twice with 0 °C CMF-PBS (Ca- and Mg-free phosphate-buffered saline: 0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 1 mM KH_2PO_4, pH 7.4). Three to four milliliters of 0 °C P buffer (0.5 M NaCl, 5 mM magnesium acetate, 10 mM Tris, pH 7.0, 0.5% Nonidet P-40, and 1% sodium dextran sulfate) was added to each flask to lyse the cells. For some polysome preparations, P2 buffer (0.12 M NaCl, 5 mM magnesium acetate, 10 mM Tris, pH 7.0, 0.5% Nonidet P-40, and 1% sodium dextran sulfate) was used instead of P buffer. After 10 min at 0 °C, Tween-40 (polyoxyethylene sorbitan monopalmitate; Sigma, St. Louis, MO) and deoxycholine (Matheson, East Rutherford, NJ) were each added to a final concentration of 0.5%. The cell lysates were then transferred to 30-mL Corex centrifuge tubes (Corning Glass Works, Corning, NY) and spun at 10000 rpm (~16000*g*) for 10 min at 2 °C in a Sorval centrifuge using an HB-4 rotor. The supernatant solution containing the polysomes was layered over 3 mL of 30% (w/v) sucrose (Schwarz/Mann ultrapure, Orangeburg, NY) in P buffer and spun in a Beckman Ti50 rotor for 3 h at 45000 rpm (maximum 185000*g*), 2 °C. The clear polysome pellets were resuspended in Tris–SLS buffer (0.1 M NaCl, 1 mM EDTA, 10 mM Tris, pH 7.0, and 0.5% SLS) and repeatedly extracted with PCIA (phenol–chloroform–isoamyl alcohol, 25:24:1). After extraction, the polysomal RNA was ethanol precipitated, pelleted in the HB-4 rotor at 10000–12500 rpm (~16000–25000*g*), and resuspended in oligo(dT) binding buffer (0.5 M NaCl, 10 mM Tris, pH 7.0, and 0.5% SLS).

Oligo(dT)–cellulose (T-3, Collaborative Research, Waltham, MA) column chromatography was modified from the method of Aviv & Leder (1972). Polysomal RNA was incubated for 2 min at 60 °C and passed through the oligo(dT) column at room temperature in binding buffer. After extensive washing with binding buffer, poly(A)-containing polysomal RNA was eluted with 10 mM Tris, pH 7.0, and 0.5% SLS. The poly(A) RNA was again recycled through the column, and the material bound on this second cycle was precipitated with ethanol and resuspended in the appropriate hybridization buffer. ^3H-Labeled polysomal RNA was prepared as above, except that cells were incubated before harvesting in growth medium containing [^3H]uridine (Schwarz/Mann, Orangeburg, NY; [5-^3H]uridine >20 Ci/mM) at a concentration of 100 μCi/mL. Hamster embryo polysomal RNA was prepared from 13-day embryos by placing the embryos directly into 0 °C P buffer, homogenizing with a Teflon–glass motorized homogenizer, and incubating at 0 °C for 10 min. Tween-40 and deoxycholate were both added to a concentration of 0.5%, and the nuclei and unlysed debris were pelleted at 11000 rpm (~20000*g*) for 15 min in a Sorval HB-4 rotor at 2 °C. Polysomes were obtained from the supernatant as described above. Absorption ratios of 260 nm/280 nm greater than 1.95 and of 260 nm/230 nm greater than 2.25 were routinely ob-

tained, and rRNA contamination of poly(A) mRNA was minimal, as confirmed by the absence of a 28S rRNA peak on sucrose gradients or 2.5% polyacrylamide gels. RNA concentrations were determined by measuring the absorption at 260 nm, assuming that 1.0 OD_{260} = 40 μg of RNA.

Preparation of Nuclei and Purification of Nuclear RNA. SHE cells and BP6T cells were grown in 490-cm^2 plastic roller bottles (Corning Glass Works, Corning, NY) as described above. In midlog phase, the monolayers were washed twice with CMF-PBS, trypsinized with 0.1% trypsin in CMF-PBS, pelleted, and resuspended in either P or P2 buffer without Nonidet P-40. The cells were lysed by the addition of Nonidet P-40 to 0.5%, and the nuclei were pelleted in a Sorval centrifuge at 5000 rpm (~4000*g*) for 15 min at 2 °C, using the HB-4 rotor. For some preparations, nuclei were resuspended in magnesium–sucrose buffer (0.3 M sucrose, 10 mM Tris, pH 7.6, and 5 mM magnesium acetate) and repelleted at 5000 rpm (~4000*g*) for 10 min at 2 °C. The nuclear pellet was processed by one of three methods. (1) A modification of the method of Holmes & Bonner (1973). Nuclei were resuspended in 7 M urea (Schwarz/Mann ultrapure, Orangeburg, NY), 0.35 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 8.0, and lysed with 0.5% SLS. This lysate was immediately extracted with an equal volume of PCIA. (2) A modification of the method of Penman (1966). The nuclear pellet was resuspended in 0.5 M NaCl, 50 mM magnesium acetate, and 10 mM Tris, pH 7.0, and treated with 50 μg/mL DNase I (Worthington, Freehold, NJ, or Sigma, St. Louis, MO). The DNase I was purified to remove RNase activity by fractionation on Sephadex G-50 (Holmes & Bonner, 1973) or 5′-(*p*-aminophenylphosphoryl)uridine 2′(3′)-phosphate–agarose (Maxwell et al., 1977). After 2 min at 37 °C, EDTA and SLS were added to 0.1 M and 0.5%, respectively, and the lysate was immediately extracted with PCIA. (3) The nuclei were resuspended in 0.1 M NaCl, 1 mM EDTA, and 10 mM Tris, pH 7.0, lysed with 0.5% SLS, and immediately extracted with PCIA. No significant difference in the sequence complexity of nuclear RNA obtained by any of these three extraction methods was observed.

Following the PCIA extractions, the aqueous phase was ethanol precipitated at −20 °C overnight. DNA was then spooled out on a glass rod, and the remaining RNA was pelleted in a Sorval HB-4 rotor at 10000 rpm (~16000*g*) for 50 min, 2 °C. The pellet of crude RNA was resuspended in TNM buffer (10 mM NaCl, 10 mM $MgCl_2$, and 10 mM Tris, pH 8.0), RNase-free DNase I was added to a final concentration of 50 μg/mL, and the mixture was incubated at 37 °C for 20 min. Proteinase K (E. Merck, Darmstadt, Germany) was then added to a final concentration of 100 μg/mL, and the mixture was again incubated at 37 °C for 30 min, followed by extraction with PCIA and ethanol precipitation. The pelleted total nuclear RNA was resuspended in 2 mM phosphate buffer (PB) (pH 6.8) or 0.15 M imidazole buffer (adjusted to pH 6.8 with 1 N HCl) and chromatographed on a 2.6 × 40 cm column of either Sephadex G-100 (Pharmacia, Piscataway, NJ) or Bio-Gel A-0.5 (Bio-Rad, Richmond, CA) in imidazole buffer. The exclusion peak containing the RNA was collected, ethanol precipitated, and resuspended in the appropriate hybridization buffer.

^{3}H-Labeled nuclear RNA was prepared by method 1 above, except that before harvesting, cells were incubated in growth medium containing [^{3}H]uridine (Schwarz/Mann, Orangeburg, NY; [5-^{3}H]uridine >20 Ci/mM) at a concentration of 200 μCi/mL for 10 min ("pulse"-labeled nuclear RNA) or 100 μCi/mL for 4 h ("steady-state"-labeled nuclear RNA). Polyadenylated nuclear RNA was obtained by oligo(dT)–cellulose chromatography as described for polysomal poly(A) mRNA.

Hamster embryo nuclei were prepared by resuspending in magnesium sucrose buffer the crude nuclear pellet produced during the initial stage of polysome purification [see Polysome Purification and Isolation of Poly(A) mRNA]. This suspension was then filtered through two layers of sterile cheesecloth and centrifuged at 4000 rpm (~2500*g*) for 10 min at 2 °C. The nuclear pellet was again washed by resuspension and pelleting in magnesium–sucrose buffer, followed by resuspension in one of the three nuclear lysis buffers described above. Extraction and purification of total embryo nuclear RNA was identical with that described for SHE and BP6T cell nuclear RNA.

Estimation of Polysomal RNA Half-Life. Polysomal poly(A) mRNA half-life was estimated by the method of Greenberg (1972). Log phase SHE or BP6T cells grown in 150-cm^2 T-flasks were labeled with [^{3}H]uridine (Schwarz/Mann, Orangeburg, NY; [5-^{3}H]uridine >20 Ci/mM) at a concentration of 100 μCi/mL. Under these conditions, equilibration was reached between intracellular and extracellular levels of radioactivity in 30 min and remained approximately constant up to 24 h. After labeling for various times, polysomes were prepared as described above and specific activities of the purified poly(A) mRNA or 18S and 28S rRNA from the nonpolyadenylated fraction were determined. Best-fit curves of the Greenberg equation to the data were computed.

DNA Isolation, Fractionation, and Labeling. DNA was prepared from Syrian hamster tissues or normal SHE cell fibroblasts as described (Moyzis, 1978). Single-copy DNA was prepared by two reassociations to C_0t = 250, both times collecting the unreassociated DNA by hydroxylapatite fractionation. Radiolabeled DNA was prepared by either labeling SHE cells with [^{3}H]thymidine or labeling hamster tissue DNA by a modification of the "gap translation" technique of Galau et al. (1976) (Moyzis, Bonnet, and Ts'o, unpublished experiments). Specific activities of single-copy tracers obtained by these methods are given in the appropriate figure legends.

DNA–RNA Hybridization in DNA Excess. The method of Melli et al. (1971) was used. [^{3}H]RNA was mixed with a 50000–250000-fold excess of hamster nuclear DNA in 0.12 M PB, pH 6.8, 1–5 mM EDTA, and 0.4% SLS. After denaturation at 100 °C for 3 min, samples were incubated at 60 °C; alternatively, the phosphate buffer concentration was adjusted to 0.48 M by the addition of 1 M PB and the sample was then incubated at 67 °C. Hybridizations were stopped at various C_0t values, diluted into 0.24 M PB, and rapidly frozen in a dry ice–ethanol bath. RNase resistance was assayed by incubating one-half of each sample with 50 μg/mL pancreatic RNase and 50 units/mL T_1 RNase for 1.0 h at 37 °C. Treated and untreated samples were precipitated on filters (Millipore, Bedford, MA, or Amicon, Lexington, MA) with 5% trichloroacetic acid in the presence of 100 μg/mL carrier yeast RNA. The ratio of ribonuclease resistant RNA to RNA input was determined for each sample and plotted vs. equivalent C_0t (Britten et al., 1974).

Preparation of [^{32}P]tDNA. In vitro labeled single-copy [^{32}P]DNA enriched for sequences transcribed in neoplastically transformed BP6T cells ([^{32}P]tDNA) was prepared essentially as described by Galau et al. (1976) and Hough-Evans et al. (1977). Briefly, hamster tissue single-copy [^{32}P]DNA (sp act. >4 × 10^7 cpm/μg) was incubated with a 5000-fold mass excess of total BP6T cell nuclear RNA to an equivalent RNA C_0t

of 10000. DNA–RNA hybrids, isolated by hydroxylapatite fractionation, were again incubated with a 5000-fold mass excess of BP6T cell nuclear RNA to an equivalent RNA C_0t of 6000. After fractionation on hydroxylapatite to reisolate DNA–RNA hybrids, the solution was digested with 20 μg/mL RNase A for 15 h at 37 °C in 0.05 M PB to destroy the residual RNA, including that in RNA–DNA hybrids. After the addition of SLS to 0.4%, proteinase K was then added to 50 μg/mL and the solution incubated for 2 h at 37 °C, followed by extraction with chloroform–isoamyl alcohol (24:1). The preparation was then dialyzed against 0.3 M KOH, 20 °C, for 15 h, neutralized with acetic acid, and fractionated on a Sephadex G-100 column in 3.0 M NaCl to remove small DNA fragments produced during the extensive handling of this tracer. The DNA from the G-100 exclusion peak was then passed over another G-100 column in 0.12 M PB, heated for 3 min at 100 °C, quenched in ice, and passed over a hydroxylapatite column in 0.12 M PB, 60 °C, to remove any "fold-back" or zero-time binding sequences. The DNA that did not bind to the hydroxylapatite column at this step was designated [^{32}P]tDNA. Control experiments indicated that this [^{32}P]tDNA tracer contained 0.04% fold-back DNA and no detectable repetitive DNA and had a reactivity with total 0.2-kb (kilobase) hamster tissue DNA at C_0t = 50000 of 68%.

DNA–RNA Hybridization in RNA Excess. The method of Galau et al. (1974) was used. Mixtures containing samples of RNA and small quantities of nonrepetitive radiolabeled DNA in 0.12 M PB, 1–5 mM EDTA, and 0.2–2% SLS were denatured at 100 °C for 3 min. Incubation was in one of three buffers: (1) 0.12 M PB, 1 mM EDTA, and 0.4% SLS, 60 °C, (2) 0.48 M PB, 1–5 mM EDTA, and 0.4–2.0% SLS, 67 °C, or (3) 0.12 M PB, 1 M NaCl, 1–5 mM EDTA, and 0.4–2.0% SLS, 70 °C, to the desired equivalent RNA C_0t. Mass ratios of RNA to DNA varied from 100:1 to 10000:1 in different experiments. Hybridization mixtures were diluted into 0.12 M PB and frozen in a dry ice–ethanol bath to terminate the reaction. Samples were divided, and one-half was chromatographed directly on hydroxylapatite in 0.12 M PB and 0.06–0.4% SLS to measure both DNA–DNA and DNA–RNA duplexes. The other half was incubated with 10 μg/mL RNase A for 15 h at 37 °C or with 20 μg/mL RNase A for 2 h at 55 °C in 0.02–0.05 M PB to destroy the RNA in RNA–DNA hybrids. After extraction with chloroform–isoamyl alcohol (24:1) this RNase-digested sample was then chromatographed on hydroxylapatite as above to measure DNA–DNA duplexes. In addition, reassociation of the radiolabeled single-copy tracer was monitored in the presence of yeast RNA in the exact concentration as the hamster mRNA or nuclear RNA. The fraction of radiolabeled DNA in DNA–RNA hybrid was computed as the difference between the observed binding of total duplex and the binding of RNase-treated reactions or control DNA–yeast RNA hybridizations. Both methods yielded similar results. Reaction mixtures containing [^{32}P]tDNA as tracer were assayed for DNA–RNA hybridization by diluting the sample into 0.12 M PB and 0.4% SLS and directly running a hydroxylapatite column in the same buffer. No measurement of DNA–DNA reassociation was determined because tracer self-reaction was found to be negligible. Data are corrected for the fraction of the radiolabeled DNA tracer capable of binding to hydroxylapatite at C_0t = 60000, driven with a 10000-fold excess of total sheared nuclear DNA. Various preparations of radiolabeled DNA tracers gave values from 62 to 87%, and these values were used to normalize the observed hybridization results. This value reflects the amount of radiolabeled DNA tracer under ~50 nucleotides, the minimum size for efficient binding to hydroxylapatite (Wilson & Thomas, 1973; Moyzis and Li, unpublished experiments). For all RNA excess experiments, the type of DNA sequence present in hybrids at the end of the reaction was determined by isolation of duplexes by hydroxylapatite fractionation, degradation of RNA with NaOH, and reassociation with total hamster nuclear DNA. In all cases, the isolated DNA reassociated as single-copy DNA, with no significant repetitive DNA contaminant.

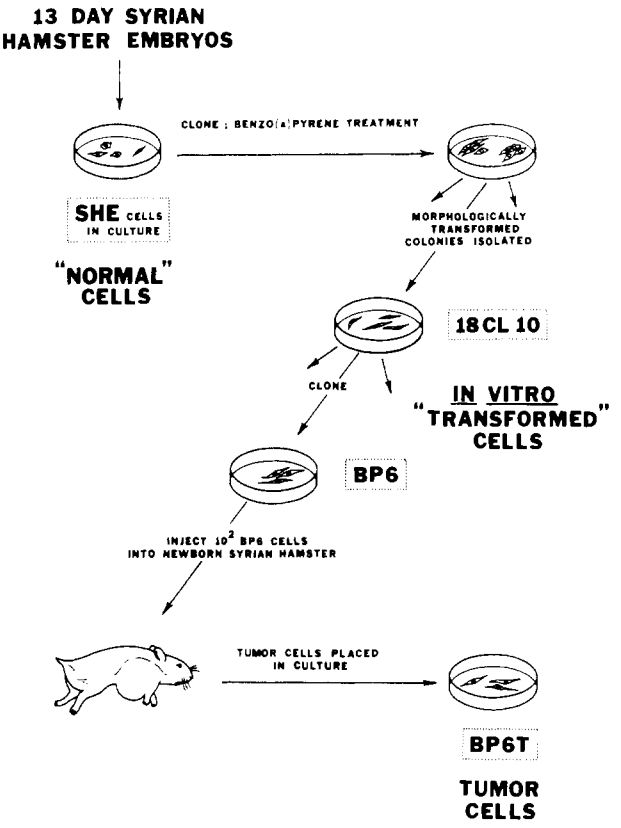

FIGURE 1: Derivation of highly tumorigenic benzo[*a*]pyrene-transformed Syrian hamster cell lines.

Results

Derivation of Highly Tumorigenic Transformed Cell Lines. A prerequisite to any biochemical or molecular study of neoplastic transformation is the acquisition of a pure population of neoplastic cells. Figure 1 diagrams the derivation of the cell lines used in this study. Primary cultures of Syrian hamster embryo cells were established as described in the Experimental Procedures section. A transformed cell line, 18 C1–10, was established from a morphologically aberrant colony induced by treatment of tertiary passage SHE cells with 10 μg/mL benzo[*a*]pyrene for 24 h (Schechtman, 1976). A normal, nonneoplastic, yet "immortal" clonal cell line, SHE 21 C1–2, was also isolated (Lin, unpublished experiments). After an additional 24 passages in culture (1:10 split ratio), subclones of transformed 18 C1–10 cells were isolated and screened for karyotypic abnormalities and increased tumorigenicity in comparison to the parent line (Moyzis, 1978). One of these subclones (BP6), after three more in vitro passages, was injected into a newborn hamster, as described in the Experimental Procedures section; the tumor cell line BP6T was derived from the tumor that formed. By using these sequential cloning and selection steps, we were ultimately able to derive a cell line (BP6T) that produces tumors in newborn Syrian hamsters following the injection of as few as 1–10 cells (Table I). This degree of tumorigenicity indicates that a high

Table I: Tumorigenicity of Normal Syrian Hamster Embryo Cells and Neoplastically Transformed Cell Lines[a]

cell type	passage no.	estimated total population doublings[b]	no. of cells inj per animal[c]	no. positive/ no. inj[c]	% positive	T_{D50}[d]
SHE	P3–P6 from initial embryo explant	9–24	10^7	0/15	0	
			5×10^6	0/6	0	$>10^7$
			10^6	0/24	0	
SHE 21 Cl-2	P21–P24 from initial cloning	96–134	10^6	0/13	0	$>10^6$
18 C1–10	P24–P26 from initial cloning and benzo[*a*]pyrene treatment	102–138	10^4	15/15	100	
			10^3	1/8	12	2700
			10^2	0/5	0	
BP6	P3 from cloning from 18 Cl–10	132–164	10^3	10/10	100	
			10^2	5/8	62	70
			10^1	0/3	0	
BP6T	PO–P5 from tumor explant	undeterminable (0–20 since tumor isolation)	10^2	16/16	100	
			10^1	16/18	89	3
			10^0	1/10	10	

[a] Tumorigenicity was assayed as described in the Experimental Procedures section. [b] Based on an estimation of three to four population doublings per passage at a 1:10 split ratio and approximately 21 to 22 population doublings per cloning process [1 cell to (2–6) × 10^6 cells per 75-cm^2 flask]. [c] 100% tumor incidence was observed for 18 Cl–10, BP6, and BP6T cells injected into newborn hamsters at numbers greater than those reported here. [d] Estimated number of cells injected to produce a 50% tumor incidence.

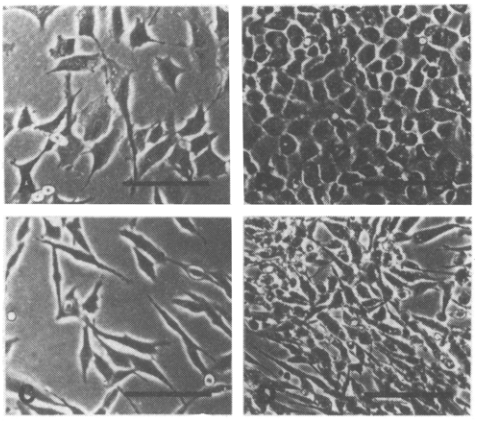

FIGURE 2: Morphology of normal and neoplastic Syrian hamster cells. Phase-contrast photomicrographs of living, normal, nonneoplastic SHE 21 C1–2 cells (A, B) and living, neoplastic BP6T cells (C, D). SHE 21 C1–2 is more "epithelioid" at confluency than most SHE cell primary cultures or clonal isolates. [For example, see Berwald & Sachs (1963), DiPaolo & Donovan (1967), or Kakunaga & Kamahora (1968).] Bars = 10 μm.

percentage, if not all, of these cells are expressing gene products associated with the tumorigenic state. This cell line also exhibits many in vitro characteristics of neoplastically transformed cells such as aberrant morphology (Figure 2), increased plating efficiency in liquid medium at low cell density, enhanced fibrinolytic activity, and anchorage-independent growth (Leavitt et al., 1977, 1978; Barrett & Ts'o, 1978; Barrett et al., 1979). Unpublished observations of Dr. E. Arnold, The Johns Hopkins University, and Dr. J. C. Barrett, National Institute of Environmental Health Sciences, indicate that this cell line is highly metastatic as well (personal communications). By comparing RNA expression in this highly tumorigenic BP6T cell line to that in (1) normal, nonneoplastic SHE cells, (2) nonneoplastic, yet immortal SHE 21 C1–2 cells, and (3) 13-day Syrian hamster embryos, it was felt that the extent of alterations in gene expression accompanying the neoplastic state could be determined. All experiments were performed on cell populations initiated at the passages indicated in Table I from cells stored in liquid nitrogen.

Half-Life of Polysomal Poly(A) mRNA. We began our analysis of RNA expression in normal and chemically transformed cells with a study of polysomal poly(A) mRNA. Figure 3A shows that the steady-state-labeled polysomal poly(A) mRNA isolated from normal SHE cells or neoplastically transformed BP6T cells has approximately the same size distribution, with a mean at 1800–2000 nucleotides. Figure 3B shows that the labeling kinetics of normal or transformed cell polysomal poly(A) mRNA are also similar, indicating that the average half-life of polysomal poly(A) mRNAs in these two cell populations is the same, ~14 h in logarithmically growing cells (Greenberg, 1972; Perry & Kelly, 1973). A similar result was obtained by Sensky et al. (1975) in their study of poly(A) mRNA half-life in normal and transformed cells.

Fraction of Steady-State-Labeled Polysomal Poly(A) mRNA Transcribed from Repetitive Sequences. The DNA excess hybridization technique originally described by Melli et al. (1971) was used to determine the distribution of steady-state-labeled polysomal poly(A) mRNA transcribed from repetitive and nonrepetitive sequences in SHE, 18 C1–10, and BP6T cells (Figure 4). Under these hybridization conditions the probability of forming a DNA–RNA hybrid depends only upon the concentration of the complementary sequence in the DNA. Approximately 22% of the mRNA in all three cell types was complementary to midrepetitive DNA sequences, while the remainder of the hybridizable mRNA (34%) appeared to be complementary to nonrepetitive DNA sequences. Forty-four percent of the poly(A) mRNA was calculated to be unable to form an RNase-resistant hybrid. This unhybridized mRNA probably consists of nonrepetitive sequence transcripts present in large abundance in these cells. At the level of DNA–RNA excess used in these experiments, putative abundant mRNA transcripts of nonrepetitive DNA would have an insufficient excess of complementary DNA sequences available for hybridization (Klein et al., 1974). Recently, an alternative explanation for this observed lack of complete hybridization in DNA excess DNA–RNA hybridizations has been proposed (Galau et al., 1977c). If the observed rate of DNA–RNA hybridization, under DNA excess conditions, is significantly slower than the rate of DNA–DNA reassociation, then most of the nonrepetitive RNA transcripts could not have hybridized with complementary DNA sequences by the final C_0t values observed in Figure 4. In either case, no significant differences are apparent between normal

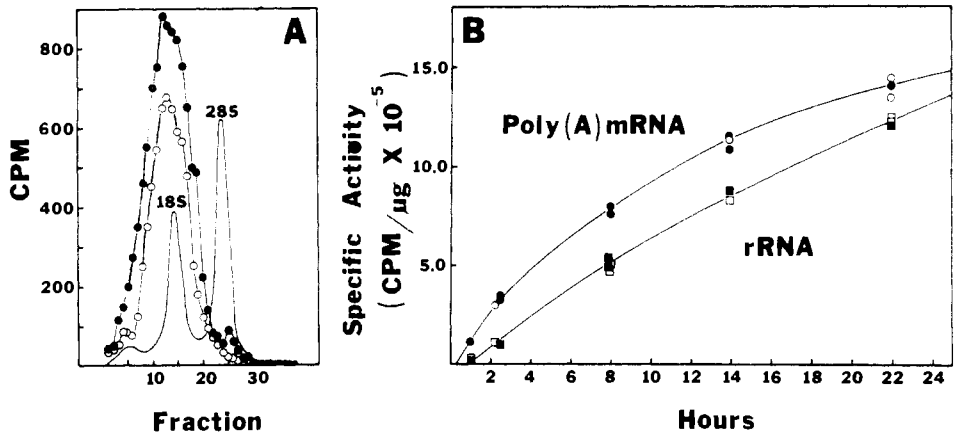

FIGURE 3: Half-life of polysomal poly(A) mRNA. (A) Size distribution of ^{3}H-labeled polysomal poly(A) mRNA from SHE cells (○) or BP6T cells (●) measured on 15–30% sucrose gradients. RNA was heated at 67 °C in 10 mM Tris, pH 7.0, 0.1 M NaCl, 1 mM EDTA, and 0.5% SLS for 3 min before layering on gradients. The sedimentation profile of nonpolyadenylated 4–5S, 18S, and 28S RNA was obtained in a separate gradient as a calibration standard (—). (B) Kinetics of labeling of polysomal poly(A) mRNA (●, ○) and 18S + 28S rRNA (■, □). Cells were labeled, during logarithmic growth, with 100 μCi/mL [^{3}H]uridine, and polysomal RNA was prepared at each time point and fractionated on oligo(dT)–cellulose (as described in the Experimental Procedures section). The specific activities of poly(A) mRNA or 18S + 28S nonpolyadenylated rRNA, fractionated on 15–30% sucrose gradients, were determined. The lines drawn are best fits of the data using the Greenberg equation (Greenberg, 1972): $A/A_\infty = 1 - e^{-(K_d+K_t)(t)}$ where A_∞ = specific activity at $t \rightarrow \infty$, A = specific activity at time t, $K_d = \ln 2/T_d$ where T_d = population doubling time, and $K_t = \ln 2/T_{1/2}$ where $T_{1/2}$ = half-life of the particular RNA species. For SHE cell rRNA (□) and BP6T rRNA (■) the parameters used are $K_d = 0.042$ h^{-1} ($T_d = 16.5$ h), $K_t = 0$, and a time lag of 1 h for the appearance of the RNA in polysomes. For SHE cell poly(A) mRNA (○) and BP6T poly(A) mRNA (●) the parameters used are $K_d = 0.042$ h^{-1} ($T_d = 16.5$ h), $K_t = 0.0495$ h^{-1} ($T_{1/2} = 14$ h), and a time lag of 0.3 h for the appearance of the RNA in polysomes. A two-component fit to the data is also possible with the more rapidly turning over poly(A) mRNA component having $K_t = 0.173$ h^{-1} ($T_{1/2} = 4$ h).

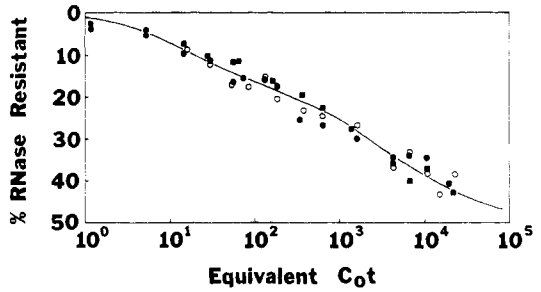

FIGURE 4: Hybridization of normal and transformed cell ^{3}H-labeled polysomal poly(A) mRNA to an excess of hamster nuclear DNA. ^{3}H-Labeled polysomal poly(A) mRNA from SHE cells (○), 18 C1–10 cells (■), or BP6T cells (●) was hybridized to a 50 000–250 000-fold excess of hamster nuclear DNA in 0.12 M PB (phosphate buffer), 1–5 mM EDTA, and 0.4% SLS or 0.48 M PB, 1–5 mM EDTA, and 0.4% SLS. RNase resistance was determined by incubation with RNase A and RNase T_1 as described in the Experimental Procedures section. The data have been fit with two components by using a modified Morrow equation (Morrow, 1974; Smith et al., 1975): $R/R_0 = (1 + kC_0t)^{-0.45}$ where R_0 = initial RNA concentration and R = fraction of RNA single stranded. The two components are (1) middle-repetitive sequence transcripts, 22% of the poly(A) mRNA, $k = 0.167$, and (2) single-copy sequence transcripts, 34% of the poly(A) mRNA, $k = 4.4 \times 10^{-4}$; 44% of the poly(A) mRNA was assumed to be unable to form an RNase-resistant hybrid under these particular DNA excess conditions. Equivalent C_0t is C_0t (the product of initial concentration and time in mole second per liter) corrected for the acceleration of hybridization rate by increased salt concentrations (Britten et al., 1974).

and neoplastically transformed cells, implying that no gross quantitative changes that would imbalance the relative amount of repetitive and single-copy transcripts have occurred during neoplastic transformation in this system.

Sequence Complexity of Polysomal Poly(A) mRNA. The technique of Galau et al. (1974) was used to determine the nonrepetitive sequence complexity of polysomal poly(A) mRNA in normal and neoplastically transformed cells. This method is a more sensitive means of determining the extent of transcription of low-frequency classes of RNA in a given cell or organ as compared to determinations based on the reassociation rate of RNA with its cDNA transcript (Bishop et al., 1974; Meyuhas & Perry, 1979). The presumed functional significance of low-frequency classes of RNA has been suggested by Galau et al. (1977b). Figure 5 shows that normal SHE cell, transformed 18 C1–10 cell, and BP6T tumor cell polysomal poly(A) mRNA all saturate 0.65 ± 0.025% of the ^{3}H-labeled normal cell single-copy DNA tracer, indicating a complexity of 2.7×10^7 nucleotides or ~13 500 genes of 2000-nucleotide average size (see Table II). Total polysomal poly(A) mRNA from 13-day hamster embryos saturates at least twice this amount of DNA (Figure 5). If one mixes normal and transformed cell poly(A) mRNAs, the percent saturation is a measure of the difference in sequence complexity of the two cell types (Grouse et al., 1972; Chikaraishi et al., 1978). Increased saturation values would result if there were partial nonhomology of the RNA sequences present in the two cell types. At the extreme upper limit, if all the mRNAs were different, the saturation value would be additive. Alternatively, if all the mRNAs are the same, no increase in the saturation value would be observed. When SHE and BP6T cell mRNAs were mixed, no increased hybridization to the single-copy DNA tracer was observed (Figure 5).

Size of Total Nuclear RNA and Poly(A) Nuclear RNA from Normal and Neoplastic Cells. In view of the above findings that polysomal poly(A) mRNA from normal or neoplastic cells exhibited no observable qualitative or quantitative differences, we continued our investigation by examining nuclear RNAs in these same cell types. After cells were labeled for 4 h with [^{3}H]uridine, nuclear RNA was isolated by a modification of the method of Holmes & Bonner (1973). Figure 6 shows the size distribution of total nuclear RNA from normal SHE cells or neoplastic BP6T cells fractionated on a calibrated Sepharose CL-2B column. A large percentage of nuclear RNA obtained from both cell types was excluded on this column and therefore behaved like RNA larger than ~8000–10 000 nucleotides. As shown in Figure 6, this excluded material consisted largely of poly(A)-containing nuclear RNA. When the poly(A) nuclear RNA was preheated to 100 °C for 1 min before application to the column, however, the

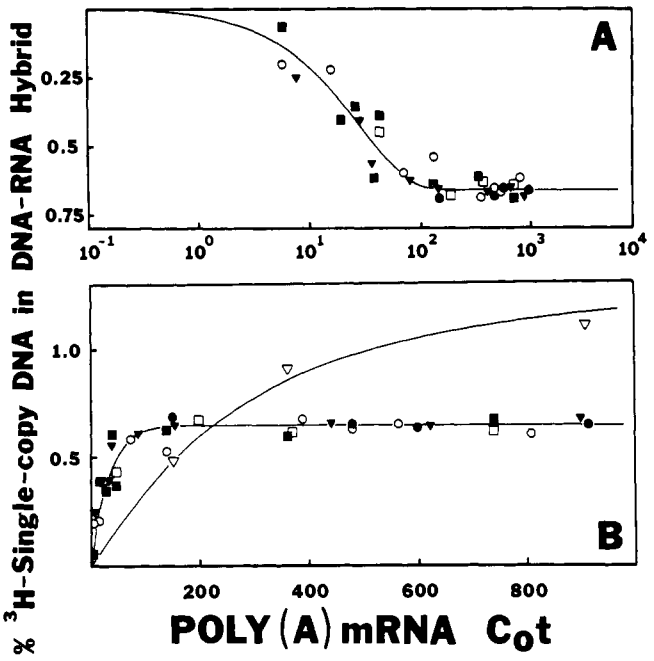

FIGURE 5: Hybridization of ^{3}H-labeled single-copy DNA to excess polysomal poly(A) mRNA. ^{3}H-Labeled single-copy DNA isolated from SHE cells (sp act. 4.8×10^5 cpm/μg) or labeled in vitro with *E. coli* DNA polymerase I, using hamster tissue single-copy DNA as a template (sp act. $>1.0 \times 10^7$ cpm/μg), was incubated with polysomal poly(A) mRNA obtained from SHE cells (○), SHE 21 C1-2 cells (□), 18 C1-10 cells (■), BP6T cells (●), or a 50:50 mixture of SHE and BP6T cell mRNA (▼). Hybridization was carried out in 0.12 M PB, 1 mM EDTA, and 0.4% SLS at 60 °C or 0.48 M PB, 1 mM EDTA, and 0.4% SLS at 67 °C, and aliquots were diluted at various times into 0.12 M PB and frozen in a dry ice–ethanol bath. The percent single-copy DNA in a DNA–RNA hybrid was determined by the method of Galau et al. (1974) as described in the Experimental Procedures section. Hybridization to polysomal poly(A) mRNA obtained directly from 13-day hamster embryos is also shown (▽). Generally, 250 000–500 000 cpm of ^{3}H-labeled single-copy DNA was used for each point and the RNA/DNA mass ratio varied from 100:1 to 4000:1. The line drawn through the data points obtained with cell mRNA is a single pseudo-first-order curve of the form $D/D_0 = e^{-(kC_0t)}$, where $k = 0.031$ (RNA $C_0t_{1/2} = 22$) and saturation is reached at 0.65 ± 0.025%. The curve for 13-day hamster embryo mRNA is also a single pseudo-first-order curve with $k = 0.0028$ (RNA $C_0t_{1/2} = 250$) and a saturation of 1.4%. Poly(A) mRNA C_0t is equivalent C_0t, assuming all RNAs are mRNA and corrected for the acceleration of hybridization rate by increased salt concentration (Britten et al., 1974). Data have been corrected for "hybridizability" of the [^{3}H]-DNA, as explained in the Experimental Procedures section. (A) Semilog plot (for kinetic analysis); (B) linear plot (for saturation analysis).

average size was reduced to ~2000 nucleotides. Since this brief heat treatment should not radically reduce the size of RNA (Eigner et al., 1961), the originally isolated RNA probably existed as an "aggregate" of several RNA molecules. Whether a specific "reassociation" of these separated RNAs can occur (Federoff et al., 1977) was not determined. In all cases, RNA from normal SHE cells and neoplastic BP6T cells behaved identically.

Fraction of Labeled Poly(A) Nuclear RNA Transcribed from Repetitive Sequences. Figure 7 shows that the fraction of labeled poly(A) nuclear RNA transcribed from repetitive sequences is only 11.5%, compared to 22% for polysomal poly(A) mRNA (Figure 4). This implies the selective loss of some nonrepetitive sequences during processing of nuclear poly(A) RNA to polysomal poly(A) mRNA or the partial nonhomology of these two RNA populations. Again, SHE and BP6T cells showed no significant differences in the pattern of hybridization, implying that no gross quantitative changes

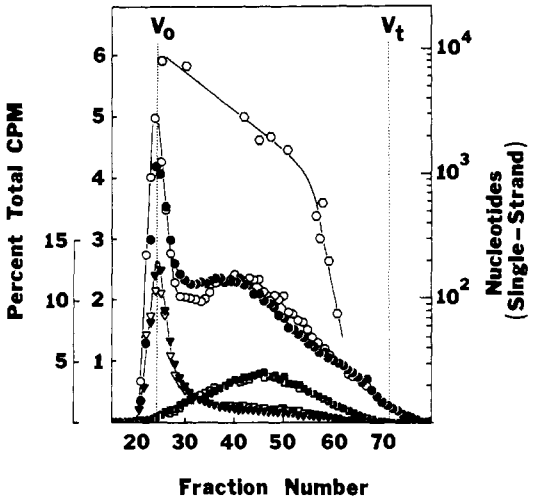

FIGURE 6: Size distribution of total nuclear RNA and poly(A) nuclear RNA. ^{3}H-Labeled nuclear RNA was isolated from normal or transformed cells as described in the Experimental Procedures section. The RNA was fractionated on a calibrated 0.9 × 50 cm Sepharose CL-2B column in 0.12 M PB. (○) SHE cell 4-h-labeled total nuclear RNA, preheated 60 °C, 2 min; (●) BP6T cell 4-h-labeled total nuclear RNA, preheated 60 °C, 2 min; (▽) SHE cell 4-h-labeled poly(A) nuclear RNA, preheated 60 °C, 2 min; (▼) BP6T cell 4-h-labeled poly(A) nuclear RNA, preheated 60 °C, 2 min; (□) SHE cell 4-h-labeled poly(A) nuclear RNA, preheated 100 °C, 1 min; (■) BP6T cell 4-h-labeled poly(A) nuclear RNA, preheated 100 °C, 1 min. Note that the scale of the poly(A) nuclear RNA has been reduced to 0.2 that of total nuclear RNA, the approximate mass ratio of poly(A) nuclear RNA to total nuclear RNA (Table II). Single-strand DNA, presized in the Model E ultracentrifuge (Studier, 1965), was used to calibrate the column (○). The exclusion volume (V_0) and the inclusion volume (V_t) are also shown. Pulse-labeled (10 min) poly(A) nuclear RNA chromatographed identically with 4-h-labeled poly(A) nuclear RNA (data not shown).

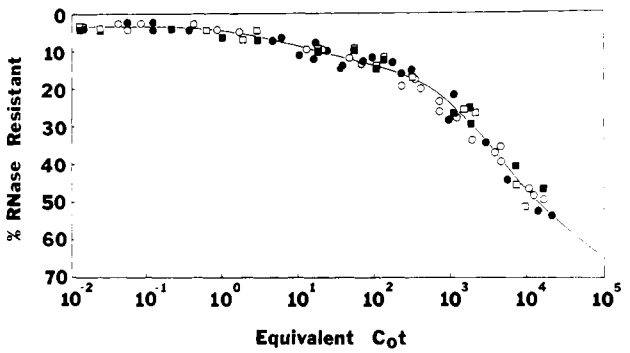

FIGURE 7: Hybridization of normal and neoplastic cell ^{3}H-labeled poly(A) nuclear RNA to an excess of hamster nuclear DNA. "Pulse" (10 min) or "steady-state" (4 h) ^{3}H-labeled poly(A) nuclear RNA obtained from SHE cells or BP6T cells was hybridized to a 50 000–250 000-fold excess of hamster nuclear DNA in 0.12 M PB, 1–5 mM EDTA, and 0.4% SLS or 0.48 M PB, 1–5 mM EDTA, and 0.4% SLS. No denaturation before isolation on oligo(dT) was attempted, so this poly(A) nuclear RNA was the "aggregated" material excluded on Sepharose CL-2B (Figure 6). RNase resistance was determined by incubation with RNase A and RNase T_1, as described in the Experimental Procedures section. The data have been fit with two components by using a modified Morrow equation as described in the legend to Figure 4. The two components are (1) middle-repetitive sequence transcripts, 11.5% of the nuclear poly(A) RNA, $k = 0.36$, and (2) single-copy sequence transcripts, 60% of the nuclear poly(A) RNA, $k = 4.4 \times 10^{-4}$; 3.5% of the RNA was "instantaneously" RNase resistant, presumably due to the presence of inverted sequence repetitions. (○) Pulsed-labeled SHE cell poly(A) nuclear RNA; (□) steady-state-labeled SHE cell poly(A) nuclear RNA; (●) pulse-labeled BP6T cell poly(A) nuclear RNA; (■) steady-state-labeled BP6T cell poly(A) nuclear RNA.

that would imbalance the relative amount of repetitive and single-copy transcripts have occurred during neoplastic

transformation in this system.

Sequence Complexity of Total Nuclear RNA. The sequence complexity of nonrepetitive DNA transcripts in total nuclear RNA was determined by using the method of Galau et al. (1974) as described above for poly(A) mRNA. In addition to being a more sensitive assay of total sequence complexity than the cDNA technique (Bishop et al., 1974; Meyuhas & Perry, 1979), this method also allows one to measure the nonrepetitive complexity of total nuclear RNA rather than just polyadenylated RNAs.

As shown in Figure 8, when total radiolabeled normal cell single-copy DNA was incubated to high RNA C_0t values with total nuclear RNA from either normal SHE cells or neoplastic BP6T cells, an apparent plateau was reached when 2.8 ± 0.17% of the DNA was in a DNA–RNA hybrid. A mixture of normal and neoplastic cell nuclear RNA also drove the reaction to an apparent saturation at 2.8 ± 0.12%. This implies extensive homology of nuclear RNAs in these two cell types. In comparison, nuclear RNA obtained from total 13-day hamster embryos had an extrapolated saturation of at least 10% of the nonrepetitive DNA (Figure 8). BP6T nuclear RNA sequences hybridized slightly faster, however, to their complementary DNA sequences than did normal SHE cell nuclear RNA sequences. This result indicates that the mass fraction of nonrepetitive nuclear RNA transcripts driving the hybridization is larger in BP6T cells than in SHE cells (see Table II). This difference could result from either an increase in the absolute number of nonrepetitive RNA transcripts per nucleus in BP6T cells or a decrease in the absolute number of repetitive sequence transcripts (such as rRNA precursors). We have evidence that the predominant sites of rRNA synthesis have been shifted to different chromosomes in BP6T cells (Moyzis, 1978), and therefore slight imbalances in the absolute concentration of rRNA precursors might seemingly be expected in this cell line. The total amount of nuclear RNA obtained from SHE or BP6T cells, however, is not significantly different. In addition, the fraction of poly(A) nuclear RNA in BP6T cells is in fact lower than that of SHE cells (Table II). The observed increased rate of hybridization of BP6T nuclear RNA sequences may be tentatively attributed, therefore, to an actual increase in the absolute number of these sequences per nucleus.

The apparent plateau reached at RNA C_0t = 10000 (Figure 8) for hybridizations driven by nuclear RNAs obtained from cultured cells does not rule out the possibility that increased hybridization would be obtained at much higher RNA C_0t values. A small increase in the slope of the "plateau" would have been difficult to detect. For example, another interpretation of the hybridization of normal cell nuclear RNA to radiolabeled single-copy DNA is shown in Figure 8, in which one RNA component saturates 2.3% of the DNA (RNA $C_0t_{1/2}$ = 1450), while a much rarer RNA component saturates an additional 1.4% of the DNA (RNA $C_0t_{1/2}$ = 29 000). This result would be expected if the majority of the cells were transcribing a particular set of sequences while a minor fraction of cells in the population (say 1 out of every 10–20) were transcribing additional sequences. While this interpretation seems possible for the presumably mixed population of cells found in early passage SHE cell cultures, it seems less likely to be true in clonal lines such as normal, nonneoplastic SHE 21 C1–2 cells and neoplastic BP6T cells. Alternatively, even maximally "repressed" genes in mammalian cells may have a low level of transcriptional readout, as has been suggested in *Escherichia coli* (Hahn et al., 1977). Because it is difficult technically to reach RNA C_0t values greater than 30000 (due to the availability, solubility, and stability of nuclear RNA), distinguishing between these various possibilities is difficult at present. However, sequences present at a concentration greater than 1 copy/10 cells should have hybridized by the final RNA C_0t values obtained (see Table II). BP6T cells

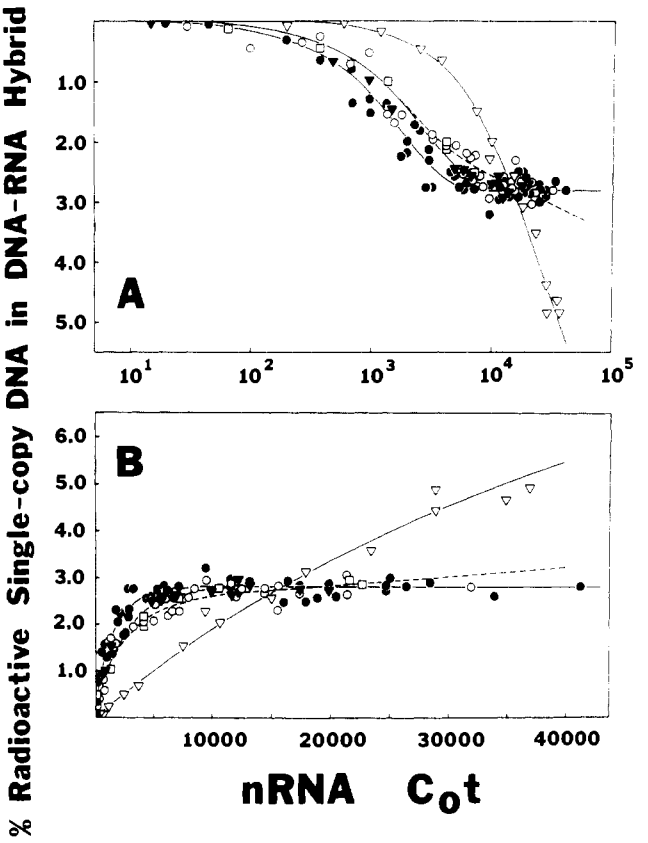

FIGURE 8: Hybridization of radioactive single-copy DNA to excess total nuclear RNA. ^3H- or ^{32}P-labeled hamster tissue single-copy DNA (labeled in vitro with *E. coli* DNA polymerase I to specific activities of 2×10^6 to $>4 \times 10^7$ cpm/μg) was incubated with total nuclear RNA obtained from SHE cells (○), SHE 21 C1–2 cells (□), BP6T cells (●), a 50:50 mixture of SHE and BP6T cells (▼), or directly from 13-day hamster embryos (▽). Hybridization was carried out in either 0.48 M PB, 1–5 mM EDTA, and 0.4–2.0% SLS at 67 °C or 1 M NaCl, 0.12 M PB, 1–5 mM EDTA, and 0.4–1.0% SLS at 70 °C. Aliquots were diluted into 0.12 M PB at various times and frozen in a dry ice–ethanol bath. The percent single-copy DNA in a DNA–RNA hybrid was determined by the method of Galau et al. (1974) as described in the Experimental Procedures section. Generally, 100 000–500 000 cpm of radioactive single-copy DNA was used for each point and the RNA/DNA mass ratio varied from 1000–10 000:1. The line drawn through the data points obtained with BP6T cell nuclear RNA is a single pseudo-first-order curve with $k = 5.7 \times 10^{-4}$ (RNA $C_0t_{1/2}$ = 1200) and a saturation of 2.8 ± 0.17%. The line drawn through the data points obtained with SHE or SHE 21 C1–2 cell nuclear RNA is a single pseudo-first-order curve with $k = 3.8 \times 10^{-4}$ (RNA $C_0t_{1/2}$ = 1850) and a saturation of 2.8 ± 0.17%. An alternate interpretation for the hybridization of radioactive single-copy DNA to normal cell nuclear RNA is also shown (- - -). It consists of two components, one with $k = 4.6 \times 10^{-4}$ (RNA $C_0t_{1/2}$ = 1450) that saturates 2.3% of the radioactive tracer and a second with $k = 2.4 \times 10^{-5}$ (RNA $C_0t_{1/2}$ = 29000) that saturates an additional 1.4%. The curve drawn through the data points obtained with 13-day hamster embryo total nuclear RNA is a pseudo-first-order curve with $k = 1.87 \times 10^{-5}$ (RNA $C_0t_{1/2}$ = 37 000) and a predicted saturation of ~10%. The hybridization of radioactive single-copy DNA to excess polysomal poly(A) mRNA (from Figure 5) is also shown (···). nRNA C_0t is equivalent nuclear RNA C_0t, corrected for the acceleration of hybridization rate by increased salt concentration (Britten et al., 1974). The data have been corrected for the "hybridizability" of the DNA tracer, as explained in the Experimental Procedures section. (A) Semilog plot (for kinetic analysis); (B) linear plot (for saturation analysis).

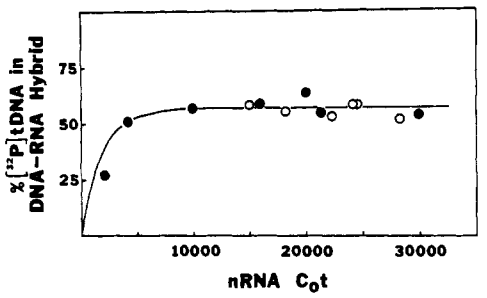

FIGURE 9: Hybridization of [^{32}P]tDNA to excess total nuclear RNA from normal or neoplastic cells. A single-copy [^{32}P]DNA tracer enriched for sequences transcribed in neoplastically transformed BP6T cells ([^{32}P]tDNA) was purified as described in the Experimental Procedures section. This [^{32}P]tDNA tracer (sp act. $>4 \times 10^7$ cpm/μg) was incubated with total nuclear RNA obtained from transformed BP6T cells (●) or normal SHE cells (○). Hybridization was carried out in 1 M NaCl, 0.12 M PB, 5 mM EDTA, and 1.0% SLS, 70 °C, and assayed by hydroxylapatite chromatography in 0.12 M PB and 0.4% SLS, 60 °C. Approximately 2500 cpm (~60 pg) of [^{32}P]tDNA was used for each column assay. The line drawn through the data points is a single pseudo-first-order curve with $k = 5.7 \times 10^{-4}$ (RNA $C_0t_{1/2} = 1200$) and a saturation of 57 ± 3.2%, the best fit for all the data points, assuming a hybridization rate equal to that obtained in Figure 8 for the hybridization of BP6T cell nuclear RNA with radiolabeled total single-copy DNA. nRNA C_0t is equivalent nuclear RNA C_0t, corrected for the acceleration of hybridization rate by increased salt concentration (Britten et al., 1974). The data have been corrected for "hybridizability" of the DNA tracer, as explained in the Experimental Procedures section.

produce tumors upon the injection of 1–10 cells into newborn Syrian hamsters; hence, sequences present at a level below 1 copy/10 cells, if they exist, are not meaningful physiologically for the expression of malignancy in this system.

An attempt was made to improve the resolution of these experiments by purifying a "cell type" specific radiolabeled tracer (Galau et al., 1976; Hough-Evans et al., 1977). As described in the Experimental Procedures section, single-copy [^{32}P]DNA was hybridized twice with excess BP6T cell total nuclear RNA to produce an enrichment for sequences transcribed in neoplastically transformed BP6T cells ([^{32}P]tDNA). As shown in Figure 9, total nuclear RNA from BP6T cells saturated 57.9 ± 3.5% of this [^{32}P]tDNA tracer, indicating a 20-fold enrichment for DNA sequences transcribed in transformed cells. Nuclear RNA obtained from normal SHE cells saturated 56.1 ± 2.6% of this same [^{32}P]tDNA tracer (Figure 9), a difference that is not statistically significant. The results of the experiments presented in Figures 8 and 9 show that the vast majority of the nuclear RNAs transcribed in neoplastic BP6T cells are identical with nuclear RNAs present in nontransformed SHE cells.

Discussion

An important aspect of this study was the development of a neoplastic cell line which is tumorigenic upon the injection of as few as 1–10 cells. One other transformed cell line, a 1-β-D-arabinofuranosylcytosine-transformed line, also of Syrian hamster origin, has been reported to exhibit this high degree of tumorigenicity (Benedict et al., 1975). To our knowledge, none of the previous studies of RNA sequence complexity in transformed cells have been conducted with highly tumorigenic lines (Grady & Campbell, 1973, 1975; Williams et al., 1977; Getz et al., 1977; Rolton et al., 1977). While in some animal systems there may be valid reasons (e.g., immunological rejection) for injecting 10^5–10^7 cells in order to produce a tumor, the possibility remains that there is but a single tumorigenic cell present in a population of 10^5–10^7 nontumorigenic cells. We do not know of a gross biochemical technique sensitive enough to detect specific molecular changes in a single tumorigenic cell present in a population of 10^5–10^7 nontumorigenic cells. This is not a trivial point, since "neoplastic-specific" RNAs present in even 1 cell out of 100 would not be detected with current nucleic acid hybridization methods. The development of BP6T was essential, therefore, for a meaningful investigation of the molecular correlates of neoplasia.

Table II summarizes the information we have obtained to date comparing RNA expression between normal SHE and neoplastically transformed BP6T cells. While some of the computations presented should be regarded as only rough approximations (for example, the number of molecules of each mRNA or nuclear RNA present per cell), most are sufficiently accurate to serve as a useful description of RNA expression in these cells. It is obvious that Syrian hamster cells in culture, like other reported mammalian cells (Bishop et al., 1974; Birnie et al., 1974; William & Penman, 1975; Getz et al., 1976; Jacquet et al., 1978), transcribe a relatively small portion of their single-copy DNA. The amount of transcription into poly(A) mRNA, for example, is only 0.65% of the total single-copy sequence complexity. While this number is small, it still implies that ~13 500 "genes" of 2000-nucleotide average size are functionally expressed in these cells. The amount of transcription into total nuclear RNA is 4.3 times greater than into poly(A) mRNA (2.8%/0.65%), however, a result similar to that reported in other systems (Getz et al., 1975; Hough et al., 1975; Bantle & Hahn, 1976; Chikaraishi et al., 1978). To what extent this increased complexity of nuclear RNA reflects the processing of a larger initial transcript to produce the final mRNA (Darnell et al., 1973; Darnell, 1978) or the expression of "regulatory" RNAs specific to the nucleus (Britten & Davidson, 1969; Robertson & Dickson, 1975; Davidson et al., 1977; Davidson & Britten, 1979) is unknown at present.

From the observed rates of hybridization of RNAs with their complementary DNAs, one can estimate the mass fraction of RNA "driving" the reaction and the relative abundance of these "complex class" RNAs (see Table II). These calculations indicate that the complex class poly(A) mRNAs are each present in ~17 copies/cell. The complex class nuclear RNAs are less abundant, each present in ~2–3 copies/nucleus. A minor difference in the rate of hybridization between neoplastic or normal cell nuclear RNA with radiolabeled single-copy DNA was observed (Figure 8, Table II). This implies that the absolute concentration of sequences may vary slightly between these two cell types, even though the total sequence complexity of nuclear RNA was indistinguishable. The significance of this difference for the expression of malignancy is difficult, at present, to evaluate. The major conclusion, therefore, of our results summarized in Table II is that none of the measured parameters of RNA expression vary significantly between normal and neoplastic cells. In contrast to the results obtained with SHE and BP6T cells, the amount of transcription observed in 13-day hamster embryos is significantly higher (Table II), presumably reflecting the diverse population of differentiated cells present at that stage of development. This huge increase in the transcriptional complexity found in hamster embryos as compared to that in cultured cell lines is consistent with the idea that all cells transcribe certain "housekeeping" genes, yet the total additional information needed to produce a differentiated organism is, indeed, immense (Galau et al., 1976; Hough-Evans et al., 1977; Caplan & Ordahl, 1978).

Table II: Comparison of RNA Expression in Normal and Neoplastically Transformed Cells

	SHE cells	BP6T cells	SHE cells + BP6T cells	13-day hamster embryos
fraction of polysomal RNA containing poly(A)[a]	0.04 ± 0.007	0.042 ± 0.01		0.05 ± 0.014
poly(A) mRNA half-life[b]	14 h	14 h		
fraction of poly(A) mRNA transcribed from repetitive sequences[c]	0.22	0.22		
fraction of single-copy DNA complementary to poly(A) mRNA[d]	0.0065 ± 0.00025	0.0065 ± 0.00025	0.0065 ± 0.00025	~0.014
base sequence complexity of single-copy DNA transcribed into poly(A) mRNA[e]	2.7×10^7 nucleotides	2.7×10^7 nucleotides	2.7×10^7 nucleotides	~5.8×10^7 nucleotides
fraction of poly(A) mRNA driving reaction[f]	0.63	0.63		~0.12
no. of molecules per high complexity sequence per cell[g]	17	17		~1–2
fraction of undenatured pulse-labeled or steady-state-labeled nuclear RNA containing poly(A)[a]	0.215 ± 0.035	0.16 ± 0.03		
fraction of poly(A) nuclear RNA transcribed from repetitive sequences[h]	0.115	0.115		
fraction of single-copy DNA transcribed into nuclear RNA[i]	0.028 ± 0.0017 (alternately, component 1 0.023, component 2 0.014)	0.028 ± 0.0017	0.028 ± 0.0012	~0.10
base sequence complexity of single-copy DNA transcribed into nuclear RNA[e]	1.16×10^8 nucleotides (alternately, component 1 9.6×10^7, component 2 5.8×10^7)	1.16×10^8 nucleotides	1.16×10^8 nucleotides	~4.16×10^8 nucleotides
fraction of nuclear RNA driving reaction[f]	0.048 (alternately, component 1 0.05, component 2 0.0015)	0.074		~0.008
no. of molecules per high complexity sequence per nucleus[g]	2 (alternately, component 1 2.5, component 2 0.12)	3		~0.09

[a] Moyzis, Grady, and Li (unpublished experiments). [b] From Figure 3. [c] From Figure 4. [d] From Figure 5. [e] Computed as follows (example): (6.6×10^9 nucleotides per haploid genome) × (0.63 fraction of genome single-copy) = 4.16×10^9 nucleotides single-copy DNA per genome (Moyzis, 1978; Moyzis, Bonnet, and Ts'o, unpublished experiments) and (4.16×10^9 nucleotides single-copy DNA per genome) × (0.0065 fraction transcribed) = 2.7×10^7 nucleotides transcribed into poly(A) mRNA. [f] Computed by comparing to the observed rate of hybridization of φX-174 DNA (5374 nucleotides) to an excess of cRNA ($k = 168$, $C_0t_{1/2} = 4.1 \times 10^{-3}$; Galau et al., 1977) (example for BP6T nRNA-DNA reassociation): observed $C_0t_{1/2} = 1200$ ($k = 5.7 \times 10^{-4}$), (4.16×10^9 nucleotides single-copy DNA per genome) × (0.028 fraction transcribed) = 1.16×10^8 nucleotides transcribed; predicted RNA $C_0t_{1/2}$ = (1.16×10^8 nucleotides) × (4.1×10^{-3})/(5374 nucleotides − [φX-174]) = 89, and predicted RNA $C_0t_{1/2}$/observed RNA $C_0t_{1/2}$ = 89/1200 = 0.074. Using the reassociation of globin mRNA with its cDNA transcript as a kinetic standard yielded similar results (4.0×10^5 daltons, RNA $C_0t_{1/2}$ = (1×10^{-3})-(3×10^{-4}) depending on the viscosity and/or concentration of the reaction mixture; Kleiman et al., 1977). The values given for the fraction of mRNA driving the reaction are an average of computations obtained with both kinetic standards. No correction for the size difference between the RNA driver to DNA tracer has been applied (Chamberlain et al., 1978), since, in our hands, the degradation of RNA at elevated temperatures during long incubations (Eigner et al., 1961; Moyzis and Li, unpublished experiments) is extensive enough to make this correction imprecise. [g] Assuming 2.5×10^{-12} g of nuclear RNA per cell and 10.0×10^{-12} g of cytoplasmic RNA per cell (Moyzis and Li, unpublished experiments), then (example) (2.5×10^{-12} g of nuclear RNA per cell) × (0.074 complex class transcripts) = 1.85×10^{-13} g of complex class RNA per nucleus; (1.85×10^{-13} g) × (6.023×10^{23})/(3.86×10^{10} daltons of complex class sequences) = 3 copies/nucleus. [h] From Figure 7. [i] From Figure 8.

The finding that normal and neoplastic cells exhibit extensive homology of RNA populations is consistent with our finding that a quantitative or qualitative change in the synthesis of less than 1% of the 1100–1200 major nuclear or cytoplasmic polypeptides visible on two-dimensional gels (O'Farrell, 1975) exists between normal SHE cells and neoplastic BP6T cells (Leavitt & Moyzis, 1978, and unpublished experiments). Taken together, the observation of extensive homology of polysomal poly(A) mRNA and nuclear RNA between normal and neoplastic cells as well as the extensive homology of the major polypeptides suggests that the phenotypic changes associated with neoplastic transformation by chemical carcinogens are accompanied by relatively few qualitative changes in gene expression of cells cultured in vitro. It would appear that massive derepression need not be invoked to account for the neoplastically transformed state, as previously reported

in virally transformed mouse cells (Grady & Campbell, 1973, 1975; Strand & August, 1977). While it is possible that these observed differences may reflect a fundamental difference in the mechanism of transformation by chemical or viral agents, the finding of extensive homology of poly(A) mRNA between normal and SV-40-transformed human cells by Williams et al. (1977) indicates that the issue remains unresolved.

The experiments reported here are limited in their ability to detect subtle changes in gene transcription. A reasonable estimate, based on the standard deviation observed with these RNA excess DNA saturation hybridization experiments, is that a change in 5% of the complexity of an RNA population would be difficult to detect by current methods. Recycling of the radiolabeled DNA to purify a cell type specific tracer (Galau et al., 1976; Hough-Evans et al., 1977) did not significantly alter this sensitivity, since, in general, the amount of radioactivity available to form a DNA–RNA hybrid was approximately the same for the recycled tracer, and the standard deviation of measurements was not significantly improved. For example, in the experiments presented in this paper we have used between 1.0×10^5 and 5.0×10^5 cpm/column assay, giving, at saturation, ca. 2600 cpm in a DNA–RNA hybrid for poly(A) mRNA driven reactions and 14 000 cpm in a DNA–RNA hybrid for nuclear RNA driven reactions. With current cell propagation technology, the difficulty of obtaining sufficient amounts of RNA from cells grown in vitro, in comparison to RNA obtained from whole animal sources, precluded the purification of more than $\sim 4.0 \times 10^4$ cpm of a neoplastically transformed cell type specific tracer (Figure 9). The counts per minute in a DNA–RNA hybrid at saturation, using such a tracer, was actually less than the counts per minute we are currently observing with an unrecycled single-copy DNA tracer.

The addition experiment presented in Figure 8 and the recycling experiment presented in Figure 9 are sufficiently precise, however, to indicate that neoplastic BP6T cells differ in gene expression from their normal SHE counterparts to a much smaller extent than do two adult tissues (Grouse et al., 1972; Brown & Church, 1972; Ryffel & McCarthy, 1975; Axel et al., 1976; Galau et al., 1976; Chikaraishi et al., 1978; Caplan & Ordahl, 1978; Davidson & Britten, 1979). At least 95–98% of the transcriptional complexity is identical in both cell types. Indeed, the homology of gene products may be still greater, since no differences in RNA expression were actually detected. If our sample of the 1100–1200 major polypeptides observable on two-dimensional gels (Leavitt & Moyzis, 1978, and unpublished experiments) is representative of the complexity of all cellular proteins in SHE cells and BP6T cells, then the homology of gene products may be greater than 99%.

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