Quartey-Papafio, P., Garlick, P. J., & Pain, V. M. (1980) *Biochem. Soc. Trans. 15*, 357.
Rigby, P. W., Dieckmann, M. A., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237.
Saber, M. A., Shafritz, D. A., & Zern, M. A. (1983) *J. Cell Biol. 97*, 986.
Saif, M., Tawfik, J., Ali, M. A., El-Mahrouky, S. F., Galil, N., & Galal, M. N. (1977) *J. Egypt. Med. Assoc. 60*, 731.
Sandmeyer, S. Gallis, B., & Bornstein, P. (1981) *J. Biol. Chem. 256*, 5022.
Shields, D., & Blobel, G. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2059.
Siddiqui, V. A., Goldflam, T., & Goodridge, A. G. (1981) *J. Biol. Chem. 256*, 4544.
Soliman, M. H., El-Waseef, A., Konbor, A. A., & El-Kashlein, M. (1973) *J. Hyg. Epidemiol., Microbiol., Immunol. 17*, 346.
Takahashi, S., Dunn, M. A., & Seifter, S. (1980) *Gastroenterology 78*, 1425.
Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 5201.
Tilghman, S., & Belayew, A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5254.
Villa-Komaroff, L., Efstratiadis, A., Broome, S., Lomedico, P., Tizard, F., Naber, S. P., Chick, W. L., & Gilbert, W. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3727.
Warren, K. S. (1980) *N. Engl. J. Med. 303*, 203.
Wyler, D. J., Wahl, S. M., & Wahl, L. M. (1978) *Science (Washington, D.C.) 202*, 438.
Yap, S. H., Strair, R. K., & Shafritz, D. A. (1978) *J. Biol. Chem. 253*, 4944.

5-Bromodeoxyuridine-Induced Amplification of Prolactin Gene in GH Cells Is an Extrachromosomal Event†

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ABSTRACT: Treatment of a 5-bromodeoxyuridine-resistant (brdUrd^r) and prolactin-nonproducing (Prl$^-$) subclone of GH cells with this drug led to amplification of the prolactin (Prl) gene and induced Prl synthesis. Withdrawal of the drug treatment reversed both of these processes. In normal rats, the increased Prl synthesis observed during late pregnancy and lactation does not seem to be mediated via amplification of the gene. Amplification of the Prl gene and induction of Prl synthesis can also be observed in the Prl$^-$, brdUrd-sensitive (brdUrd^s) GH cell strain. Prl gene amplification thus does not seem to be associated with the mechanism that confers the brdUrd^r phenotype to these cells. brdUrd-induced amplification of the Prl gene can be identified with the low molecular weight, extrachromosomal, supernatant DNA fraction, isolated by Hirt's method. Southern blot analysis of Hirt's supernatant DNA (undigested) from brdUrd-treated cells generated a distinct band following hybridization with [^{32}P]$_p$DNA$_{Prl}$-insert. The size of this band is greater than 23 kb but smaller than chromosomal DNA. Growth hormone (Gh) and albumin (Alb) gene sequences can be detected in the chromosomal DNA preparation but are absent in the extrachromosomal DNA prepared from Hirt's supernatant. The levels of Gh and Alb sequences are unaffected by brdUrd treatment of these cells. Results presented here suggest that in rat pituitary glands as well as in GH cells, hormonally controlled increased Prl synthesis is not caused by gene amplification. However, the brdUrd-induced expression of the Prl gene seems to be linked to the mechanism of drug-induced amplification of the Prl gene, mediated via an extrachromosomal event.

Different GH[1] (rat pituitary tumor) cells in culture synthesize and secrete different amounts of prolactin (Prl) (Brennessel & Biswas, 1980). Prl synthesis cannot be detected in the GH subclone GH_12C_1. Two 5-bromodeoxyuridine-resistant (brdUrd^r) strains were derived from GH_12C_1 by stepwise exposure of these cells to drug concentrations higher than the sublethal dose (Biswas et al., 1977). Prl synthesis can be induced in one of the brdUrd derivatives, $F_1BGH_12C_1$, and in the parent strain, GH_12C_1, following treatment of the cells with the drug (Biswas et al., 1977), whereas the second brdUrd^r derivative, $F_2BGH_12C_1$, does not synthesize Prl either in the absence or in the presence of the drug. Our recent results demonstrate that treatment of $F_1BGH_12C_1$ cells with brdUrd increases the level of Prl gene sequences in conjunction with the increased Prl production (Biswas & Hanes, 1982).

In eukaryotes, amplification of specific genes has been observed in several systems during normal cellular developmental processes in parallel with the overproduction of the gene product. The rRNA gene sequences increase several thousand fold during oogenesis in *Xenopus* (Brown & Dawid, 1968) and during macronuclear formation in *Tetrahymena* (Yao et al., 1979). Amplification of chorion protein genes during oogenesis in *Drosophila melanogaster* (Spradling & Mahowald, 1980) and amplification of actin genes during myogenesis in chicken (Zimmer & Schwartz, 1982) are two other examples of such gene amplification processes during normal development. Gene amplification is also observed in several instances in eukaryotic cells, which utilize this mechanism to resist the toxic effects of certain drugs. Overproduction of dihydrofolate reductase was observed in methotrexate-resistant mouse and

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[1] Abbreviations: GH cells, rat pituitary tumor cells in culture; Prl, prolactin; Alb, albumin; Gh, growth hormone; brdUrd, 5-bromodeoxyuridine; r, resistant; s, sensitive; Prl$^+$, prolactin-producing cells; Prl$^-$, prolactin-nonproducing cells; $_pDNA_{Prl}$, cloned (in plasmid pBR322) DNA complementary to mRNA$_{Prl}$ (Gubbins et al., 1979); $_pDNA_{Gh}$, cloned DNA complementary to mRNA$_{Gh}$ (Seeberg et al., 1977); $_pDNA_{Alb}$, cloned DNA complementary to rat mRNA$_{Alb}$ (Gorin & Tilghman, 1980); $_pDNA_{Prl}$-insert, $_pDNA_{Prl}$ sequence released by *Pst*I from the plasmid DNA.

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hamster cells (Schimke et al., 1979). Amplification of CAD (carbamoylphosphate synthetase, aspartate transcarbamylase, and dihydro-orotase) genes in response to *N*-(phosphonoacetyl)-L-aspartate in hamster cells (Wahl et al., 1979) and amplification of metallothionein-1 gene in response to cadmium treatment in mouse cells (Mayo & Palmiter, 1982) are observed. Brennand et al. (1982) isolated hypoxanthineguanine phosphoribosyltransferase (HPRT) producers from nonproducers. This reversion apparently is caused by amplification of the HPRT gene.

The phenomenon of drug-dependent Prl gene amplification and simultaneous expression of the gene in GH cells has been utilized in this investigation to answer the following questions: (1) Is increased production of Prl during normal developmental processes accompanied by Prl gene amplification? (2) Is brdUrd-induced amplification of the Prl gene related to the mechanism by which the cells acquired the drug-resistant phenotype? (3) Is amplification of the Prl gene restricted to only those subclones of GH cells in which the expression of the specific gene is suppressed by an as yet unresolved mechanism? (4) Is hormonally induced overproduction of Prl in certain Prl-producing (Prl^+) GH cells accompanied by gene amplification? (5) And finally, is brdUrd-induced Prl gene amplification in GH cells an extrachromosomal event?

Materials and Methods

GH cells are multihormone-producing rat pituitary tumor cells in culture. Isolation, growth conditions, and the properties of the different GH cell strains used in this investigation have been described previously (Brennessel & Biswas, 1980). Serum levels of Prl and growth hormone (Gh) were determined by microcomplement fixation assay (Tashjian et al., 1970).

Southern Blot Analysis of Cellular DNA. Pituitary glands from Sprague-Dawley rats (Charles River Breeding Laboratories) were dissected out, frozen in liquid N_2, and stored at −80 °C. Total-cell DNA from rat pituitary glands and from control and treated GH cell strains was isolated according to Gross-Bellard et al. (1973). DNA concentrations were determined by measurement of absorbance at 260 nm or by the spectrofluorometric method of Hinegardner (1971). Restriction endonuclease (*Hin*dIII, Bethesda Research Laboratory, Bethesda, MD) digestion was carried out under the conditions specified by the manufacturer of the enzyme. The *Hin*dIII-treated DNA was then resolved by electrophoresis on 1% agarose gels and blot transferred to Genatran 45 (D & L Filter Corp., Woburn, MA) according to the method described by Southern (1975). Conditions for subsequent baking, prehybridization, and hybridization of the DNA fragments to ^{32}P-labeled Prl probes and autoradiography have been described previously (Biswas & Hanes, 1982).

Dot Hybridization Analysis of Cell DNA for Detection of Prl Gene Sequences. The levels of Prl gene sequences in total cell DNA were determined by the dot hybridization procedure of Kafatos et al. (1979), modified by Tlsty et al. (1982). Specified amounts of genomic DNA were denatured in 0.3 N NaOH at room temperature for 15 min. An equal volume of 2 M ammonium acetate was then added to the denatured DNA solution, and aliquots were applied to parallel slots of a plastic blot assembly unit. After the entire DNA solution was soaked into the filter, the slots were washed with 1 M ammonium acetate. The entire filter was then washed, air-dried, baked, prehybridized, and hybridized with ^{32}P-labeled probes.

DNA Preparation from Hirt's Supernatant and Pellet Fractions. $F_1BGH_12C_1$ cells were grown to confluence in F10 medium in 75-cm^2 tissue-culture flasks (Corning) in the absence (control) and in the presence of brdUrd. Duration and concentration of drug treatments are indicated in the legend of each experiment. Cells were then washed with Hank's buffer 3 times and lysed under conditions described by Hirt (1967) with very gentle shaking for 15 min at room temperature. The viscous lysed cell suspension was transferred into a plastic centrifuge tube and one-fourth volume of 5 M NaCl was added and mixed by gentle rotation of the tubes. Vigorous shaking was avoided to prevent any shearing of DNA. The sample was then centrifuged at 8000 rpm for 15 min in the IEC rotor 870 in an IEC B20 refrigerated centrifuge. The supernatant was filtered through siliconized glass wool and subjected to RNase (pancreatic RNase A 50 μg/mL and T1 2 μg/mL) treatment, followed by proteinase k treatment (Biswas & Hanes, 1982). The samples were then extracted with phenol by gentle shaking, and DNA was precipitated in 2.5 volumes of absolute alcohol in the presence of 0.2 M sodium acetate, pH 7, at −20 °C for 16–18 h. The DNA precipitate was then pelleted by centrifugation at 4 °C at 10 000 rpm in the IEC rotor 870 in an IEC B20 refrigerated centrifuge for 30 min. The pellet (the high molecular weight, chromosomal DNA) obtained after removing the Hirt's supernatant (the low molecular weight, extrachromosomal DNA) fraction was subjected to identical treatment with RNase and proteinase k, followed by phenol extraction and alcohol precipitation. DNA preparations from Hirt's supernatant and pellet fractions were further characterized by electrophoresis on 1% agarose gel (Biswas & Hanes, 1982), by Southern blot analysis (Southern, 1975), and by banding in CsCl (Vinograd et al., 1963; Hirt, 1967) as described in the legends of the figures. Supernatant DNA constitutes about 0.5–1% of the total-cell DNA in control cells and about 1–3% of drug-treated cells. The significance of the somewhat higher levels of supernatant DNA observed in drug-treated cells is not established. The total amount of DNA isolated from equal numbers of control or drug-treated cells is essentially the same.

Results

Prl Synthesis and Levels of Prl Gene Sequences in Pregnant and Lactating Rats. Prl synthesis in female rats is stimulated during pregnancy and lactation. This is reflected by increased levels of serum Prl during such developmental processes in these animals. Results presented in Figure 1 (top panel) demonstrate that serum Prl levels increase significantly during the latter part of pregnancy and during lactation, whereas growth hormone (Gh) levels remain the same during these periods in pregnant and lactating rats. Southern blot analysis of pituitary DNA isolated from virgin, pregnant, and lactating rats reveals that the levels of Prl gene sequences are not altered (Figure 1, bottom, panel A) in such developmental processes during which the synthesis of a specific gene product, i.e., Prl, is significantly stimulated. The concentration of Prl gene sequences in indicated amounts of total pituitary cell DNA (40 μg) is the same in virgin (Figure 1, bottom, panel A, lane A), pregnant (Figure 1, bottom, panel A, lanes B–D), and lactating rats (Figure 1, bottom, panel A, lane E). These results suggest that observed stimulation of Prl synthesis during the latter phase of pregnancy and during lactation is not mediated via gene amplification. However, Southern blot analysis demonstrates that the level of Prl gene sequences in the GH subclone $F_1BGH_12C_1$, which synthesizes Prl only in the presence of the drug, is higher in drug-treated cells (Figure 1, bottom, panel B, lane A) than in control cells (panel B, lane B). The apparent differences between the signals (autoradiographic bands) observed with the same amounts (40 μg) of genomic DNA from rats (bottom, panel A) and from

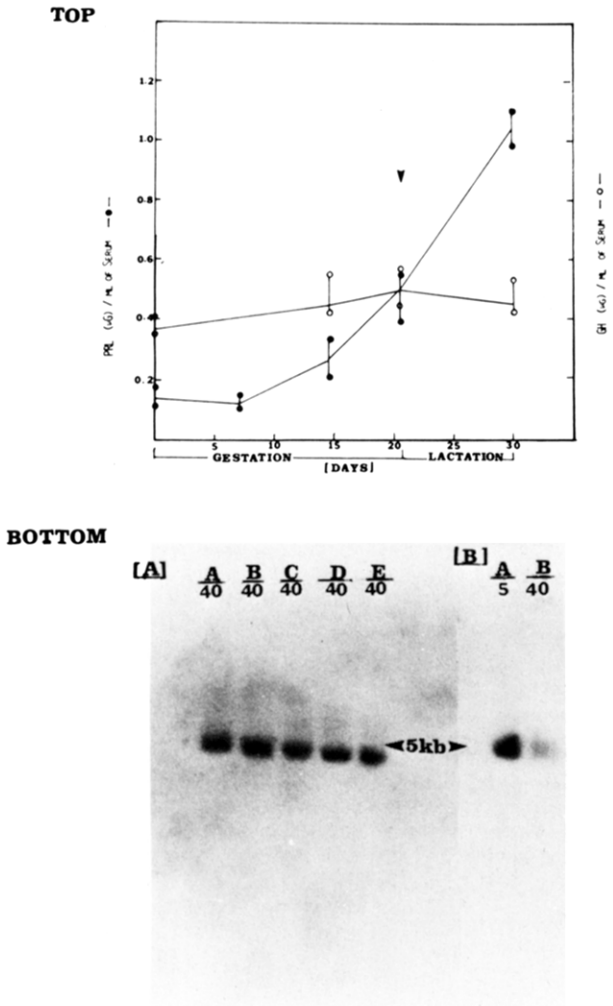

FIGURE 1: (Top) Serum Prl and Gh levels in rats during gestation and during lactation. Sprague-Dawley female rats were mated (Charles River Breeding Laboratory, Inc.) on day zero. Rats, in groups of ten, were sacrificed on the day of mating and on the 7th, 14th and 21st days of pregnancy. The fifth group was sacrificed on the 9th day of the lactation period. The arrow indicates the day on which the rats gave birth (21st day after mating). Serum Prl (●) and Gh (○) levels were determined by microcomplement fixation assay (Tashjian et al., 1970). The average values of ten determinations of serum levels of hormones are plotted. The highest and the lowest values are indicated by upper and lower points on the bar, in each group of ten determinations. (Bottom) Southern blot analysis of DNA isolated from pituitary gland of term-pregnant and lactating rats. (Panel A) Pituitary glands (ten) from each batch of rats were disected out and pooled and stored frozen at −80 °C. Total-cell DNA from the pituitary glands was isolated according to the method of Gross-Bellard et al. (1973). Indicated amounts of *Hin*dIII-digested DNA (micrograms, indicated by the numerals on top of each lane) were electrophoresed in 1% agarose slab gel, blot transferred (Southern, 1975) to Genatran 45 (D & L Filters, Woburn, MA), and probed with ^{32}P-labeled $_{p}DNA_{Prl}$-insert (2×10^6 cpm/5 mL of hybridization mix, sp act. of $[^{32}P]_{p}DNA_{Prl}$-insert 8×10^7 cpm/μg of DNA). Hybridization was carried out for 72 h, and autoradiography was for 7 days at −80 °C. Indicated amounts (micrograms) of *Hin*dIII-digested DNA from pituitary gland of virgin (lane A), 7-day pregnant (lane B), 14-day pregnant (lane C), 21-day pregnant (lane D), and 9-day lactating rats (lane E) were loaded on the gel. The arrow indicates the position of the 5-kb DNA fragment as calculated from the mobility of *Hin*dIII-digested λ DNA electrophoresed under identical conditions. (Panel B) Indicated amounts of *Hin*dIII-digested DNA from brdUrd-treated (300 μg/mL for 5 days) (lane A) and control (lane B) $F_1BGH_12C_1$ cells were loaded on 1% agarose gel and analyzed as described in panel A. $[^{32}P]_{p}DNA_{Prl}$-insert (2×10^6 cpm/5 mL of hybridization mix, sp act. of $[^{32}P]_{p}DNA_{Prl}$-insert 5×10^7 cpm/μg of DNA). Hybridization was carried out for 72 h, and autoradiography was for 7 days at −80 °C.

$F_1BGH_12C_1$ cells (panel B) are due to different hybridization conditions. Thus, no comparative estimate of the number of Prl gene copies in normal rats and in untreated $F_1BGH_12C_1$ cells may be made from these results.

brdUrd-Induced Prl Gene Amplification and Its Correlation to the Drug-Resistant Phenotype of Cells. Relative concentrations of specific gene sequences in total-cell DNA preparations can be determined by the dot hybridization technique described by Kafatos et al. (1979) and modified by Tlsty et al. (1982). We have examined the levels of Prl gene sequences by this method in total DNA isolated from different GH cell strains before and after drug treatment. ^{32}P-labeled cloned $_{p}DNA_{Prl}$-insert is used as a probe to detect Prl gene sequences and to have an estimate of the relative levels of the same in equivalent amounts of genomic DNA preparations. The dot hybridization technique generates signals (autoradiographic spots), the intensities of which seem to be proportional to the concentration of the specific DNA sequence. In confirmation with our previously reported observations (Biswas & Hanes, 1982), the results obtained by the dot hybridization technique demonstrate that brdUrd treatment of $F_1BGH_12C_1$ cells is accompanied by an increased level of Prl gene sequences, in contrast to that which can be detected in the same amounts of DNA from untreated cells. It is evident from the densities of the autoradiographic spots in Figure 2 that the concentrations of Prl gene sequences in 1, 2, and 5 μg of total-cell DNA of brdUrd-treated $F_1BGH_12C_1$ cells (300 μg/mL, for 5 days, spots 1, 2, and 3, row A; 30 μg/mL, for 10 days, spots 4, 5, and 6, row A) are higher than those in the same amounts of total-cell DNA isolated from untreated $F_1BGH_12C_1$ cells (spots 7, 8, and 9 of row A). Though dot hybridization analysis demonstrates a gross increase in Prl gene sequences in drug-treated cell DNA, the apparent differences in the intensities of the autoradiographic spots from control and drug-treated cell DNA do not seem to be as large (40-fold) as that observed in Southern blot analysis (Figure 2, bottom, panel B; Biswas & Hanes, 1982). This is possibly due to the higher sensitivity of Southern blot analysis in which hybridization with ^{32}P-labeled probes follows prior resolution of the DNA preparations. Results presented in Figure 2 (row B, spots 8 and 9) demonstrate that the concentration of the Prl gene sequences in total-cell DNA of the brdUrd^s parent strain, GH_12C_1, also increases after drug treatment at the sublethal concentration (5 μg/mL, for 10 days). Both the brdUrd^r ($F_1BGH_12C_1$) and the brdUrd^s (GH_12C_1) cells do not synthesize Prl but do so when treated with the drug at concentrations of 30–300 μg/mL in the case of the brdUrd^r strain and at sublethal concentrations (5 μg/mL) in the brdUrd^s parent GH_12C_1 strain (Biswas et al., 1977). In contrast, the level of Prl gene sequences in a different brdUrd^r subclone of GH_12C_1 ($F_2BGH_12C_1$) is not affected following brdUrd treatment of the cells (30 μg/mL, for 10 days; Figure 2, row B, spots 1–5). This brdUrd^r subclone of GH_12C_1 does not synthesize Prl either in the absence or in the presence of the drug. These results demonstrate that Prl gene amplification is not only observed in the brdUrd^r subclone, $F_1BGH_12C_1$; this phenomenon can be noticed also in the drug-treated brdUrd^s parent strain, GH_12C_1 cells, at the sublethal concentration. Thus, the observed brdUrd-induced amplification of the Prl gene in $F_1BGH_12C_1$ cannot be linked to the mechanism that conferred the drug-resistant phenotype of these cells.

GH_3 cells synthesize large amounts of Prl at the basal state, and Prl synthesis can be further stimulated by treatment of the cells with TRH (Dannies & Tashjian, 1973; Maurer et al., 1976; Evans et al., 1978). Results presented in Figure 2

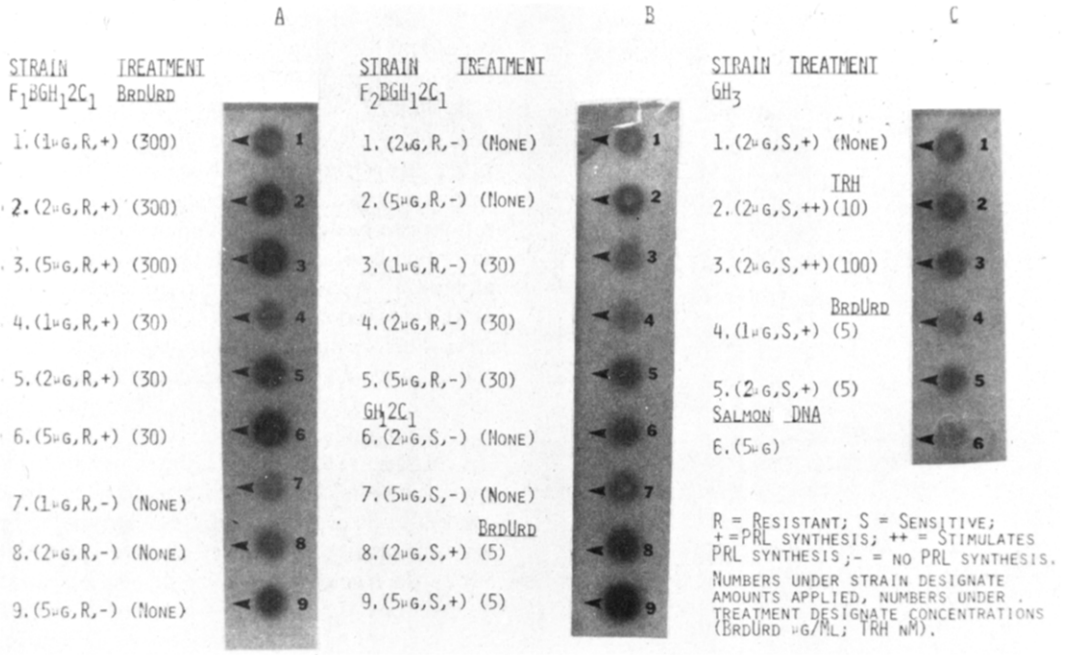

FIGURE 2: Levels of Prl gene sequences in drug-treated brdUrd^r and brdUrd^s GH cell strains. Different GH cell strains were grown in the presence or absence of brdUrd or TRH at the concentrations indicated by the numbers in parentheses (brdUrd, μg/mL; TRH, nM). $F_1BGH_12C_1$ cells were treated with 300 μg/mL for 5 days and with 30 μg/mL for 10 days. $F_2BGH_12C_1$ cells were treated with 30 μg/mL for 10 days. GH_3 and GH_12C_1 cells were treated with 5 μg/mL (sublethal) brdUrd for 10 days. GH_3 cells were treated with TRH for 3 days. Indicated amounts (μg) of DNA isolated from the control or treated cells were then denatured and spotted on Genatran 45 under conditions described under Materials and Methods. The filter disk was then prehybridized and hybridized with ^{32}P-labeled $_pDNA_{Prl}$-insert probe (1×10^6 cpm/5 mL of hybridization mixture, sp act. 1.0×10^8 cpm/μg of DNA). Hybridization was carried out for 48 h followed by washing and autoradiography for 7 days. DNA applied to each dot was made up to 5 μg with single-stranded salmon DNA.

demonstrate that the concentrations of Prl gene sequences per 2 μg of total cell DNA isolated from untreated (row C, spot 1), TRH-treated (10 nM, row C, spot 2, and 100 nM, spot), and brdUrd-treated (5 μg/mL, for 10 days, row C, spots 4 and 5) cells are similar, although Prl synthesis in TRH-treated cells is greater than that of untreated cells. These results suggest that TRH-induced stimulation of Prl synthesis in GH_3 cells is not mediated via amplification of the Prl gene. This is also in agreement with the results presented in Figure 1, which demonstrate that hormonally induced stimulation of Prl synthesis under physiological conditions such as in late pregnancy and during lactation is not accompanied by amplification of the gene. Results presented in Figure 2 suggest that Prl gene amplification by the drug brdUrd can be correlated with brdUrd-induced Prl synthesis only in those substrains of GH cells in which Prl synthesis is suppressed.

Identification of Prl Gene Amplification in Low Molecular Weight DNA Fraction of Hirt's Supernatant by Dot Hybridization. We have previously reported that induction of Prl synthesis and Prl gene amplification in Prl$^-$ $F_1BGH_12C_1$ cells can be observed only in the presence of the drug (Biswas et al., 1979; Biswas & Hanes, 1982). This is further substantiated by the results presented in Figure 3, which demonstrate that the observed increase in the level of Prl gene sequences after 120-h treatment (+B, 120 h) of the cells with brdUrd reduced to that of control cells within 144 h after withdrawal of the drug (−B, 144 h). These results suggested that the drug-induced Prl gene amplification is an unstable phenomenon and may be an extrachromosomal event.

The extrachromosomal nature of the amplified Prl gene was shown by isolation of low molecular weight, extrachromosomal DNA (from Hirt's supernatant; Hirt, 1967) separated from high molecular weight chromosomal DNA (from Hirt's pellet) followed by measurement of the concentrations of Prl, Gh, and albumin (Alb) gene sequences in DNA preparations from

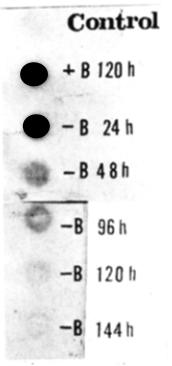

FIGURE 3: Kinetics of reversal of brdUrd-induced Prl gene amplification. One microgram of cell DNA from untreated (control) and brdUrd-treated $F_1BGH_12C_1$ cells (+B, 120 h) and from cells harvested 24, 48, 96, 120, and 144 h after withdrawal of drug treatment (−B) was spotted on Genatran 45 as described in the legend to Figure 2. The filter disks were then prehybridized and hybridized with ^{32}P-labeled $_pDNA_{Prl}$-insert (2×10^6 cpm/5 mL of hybridization mixture; sp act. 1×10^8 cpm/μg) for 48 h; the filter disk was then washed, dried, and autoradiographed at −80 °C for 7 days.

drug-treated cells by dot hybridization and by Southern blot analysis. Results presented in Figure 4 demonstrate that the observed brdUrd-induced amplification of the Prl gene can be identified in the low molecular weight extrachromosomal DNA isolated from Hirt's supernatant (Hirt, 1967) of drug-treated cells. DNA isolated from Hirt's supernatant (Figure 4B,C, S) of drug-treated cells shows higher levels of Prl gene sequences in comparison to that observed in the same amounts of pellet DNA (Figure 4B,C, P), whereas no significant amount of Gh gene sequences (Figure 4A, S) and albumin sequences (Figure 4C, S) could be detected in the supernatant of drug-treated cells. Both Gh and Alb sequences could be detected in pellet DNA (chromosomal) (Figure 4A,C, P). The relatively higher signal for Gh gene sequences over the Prl gene

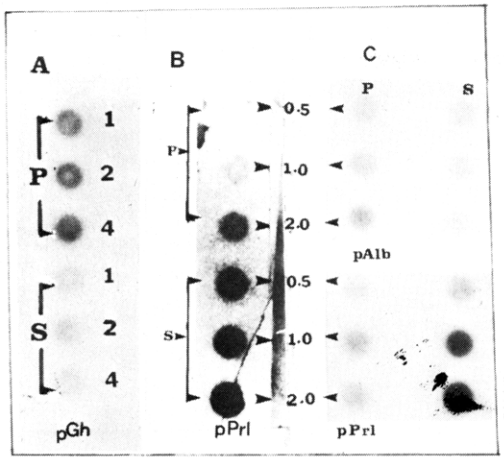

FIGURE 4: Level of Prl gene sequences in DNA preparations from Hirt's supernatant and pellet fractions of treated cells. $F_1BGH_12C_1$ cells were grown in the presence of brdUrd (300 μg/mL, 5 days). DNA from Hirt's supernatant and pellet fractions (Hirt, 1967) was isolated as described under Materials and Methods. Indicated amounts (μg) of DNA extracted from Hirt's pellet (P) and supernatant (S) were denatured in alkali and spotted on a Genatran 45 filter disk under conditions described under Materials and Methods. The filter disks were then dried, baked, prehybridized, and hybridized with a ^{32}P-labeled $_pDNA_{Gh}$-insert probe (sp act. 2×10^8 cpm/μg of DNA) (panel A), with a ^{32}P-labeled $_pDNA_{Prl}$-insert probe (sp act. 1×10^8 cpm/μg of DNA) (panel B), and with a ^{32}P-labeled $_pDNA_{Alb}$-insert probe (sp act. 8×10^7 cpm/μg of DNA) (panel C, top) under conditions described in the legend of Figure 2. 2×10^6 cpm of each probe was used per 5 mL of hybridization mix. The hybridization was carried out for 48 h, and the filter disks were then washed, dried, and autoradiographed at −80 °C for 5 days.

sequences in the same pellet DNA (chromosomal) is mainly due to the higher specific activity of the ^{32}P-labeled Gh probe (please see legend to Figure 4). These results thus suggest that brdUrd-induced amplification of Prl gene sequences in $F_1BGH_12C_1$ cells is associated with an extrachromosomal event and involves specific genes.

The extrachromosomal Hirt's supernatant DNA is prepared under conditions in which no significant shearing of DNA was reported (Hirt, 1967). Analysis of Hirt's supernatant DNA preparations from drug-treated cells, by electrophoresis on 1% agarose gels, shows a distinct ethidium bromide stained band of DNA (Figure 5, panel A, lane 1) of restricted size that is smaller than the chromosomal (Hirt's pellet) DNA. Similarly, a distinct ethidium bromide stained DNA band is noticed after electrophoresis of the same amount (1 μg) of Hirt's supernatant DNA after RNase A treatment (Figure 5, panel A, lane 2). These results demonstrate that the observed ethidium bromide stained band in the Hirt's supernatant does not contain detectable amounts of RNA. Shearing of high molecular weight chromosomal DNA would be a random process and thus would generate different size classes of comparatively lower molecular weight DNA species, resulting in a diffuse ethidium bromide stained band. Hirt's supernatant DNA moves as a very sharp and compact band and thus represents a specific size class of DNA and does not seem to be a random-shearing product of high molecular weight chromosomal DNA. Analysis of 5 times the amount of high molecular weight chromosomal DNA does not reveal the presence of a sharp DNA band of the same nature as observed in Hirt's supernatant DNA (data not shown).

Further characterization of chromosomal and extrachromosomal DNA, isolated from control and drug-treated cells by Southern blot analysis following *Hin*dIII digestion, revealed that supernatant DNA of treated cells is enriched with Prl gene sequences (Figure 5). The characteristic Prl gene sequence

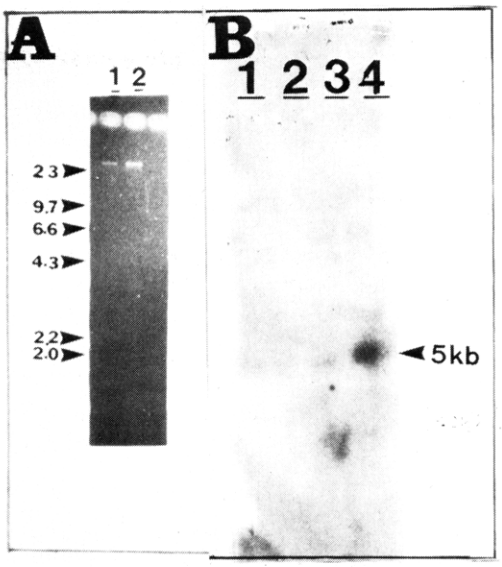

FIGURE 5: Characterization of Hirt's supernatant DNA by Southern blot analysis. (Panel A) Hirt's supernatant DNA (1 μg) from brdUrd-treated (300 μg/mL; 5 days) cells and 1 μg of the same DNA after digestion with pancreatic RNase A (50 μg/mL) were loaded on lanes 1 and 2, respectively, electrophoresed on a 1% agarose gel, and stained with ethidium bromide under conditions described previously (Biswas & Hanes, 1982). The stained DNA was then photographed under UV illumination. The numbers indicate the position of the *Hin*dIII-digested λ DNA fragments electrophoresed under identical conditions. (Panel B) DNA (10 μg each) isolated from Hirt's pellet (lane 1, control; lane 3, brdUrd treated, 300 μg/mL, 5 days) and Hirt's supernatant (lane 2, control; lane 4, drug treated) of control and drug-treated cells was digested with the restriction endonuclease *Hin*dIII (New England BioLabs) under the conditions specified by the manufacturer of the enzyme. The digested DNA fractions were then electrophoresed on 1% agarose, blot transferred to a membrane filter, washed, prehybridized, and hybridized with ^{32}P-labeled $_pDNA_{Prl}$-insert (sp act. 8×10^7 cpm/μg of DNA; 2×10^6 cpm/5 mL of hybridization mix) under conditions described previously. The arrow indicates the mobility of the 5-kb DNA fragments under the same electrophoresis conditions as calculated from the mobility of *Hin*dIII-digested λ-phage DNA.

containing the 5-kb radioactive band could be detected in all the DNA preparations from control and drug-treated cells, but autoradiographic signals generated by the same amount (10 μg) of supernatant DNA from drug-treated cells are much higher than the others. Signals observed in Prl gene sequences in control supernatant DNA (10 μg) are insignificantly small.

Analysis of Hirt's Supernatant DNA by Band Centrifugation in CsCl. Vinograd et al. (1963) reported that cellular macromolecules and viruses can be fractionated by band centrifugation in a self-generating density gradient. Hirt (1967) later utilized this method to separate viral DNA from the DNA of polyoma virus infected cells. For further characterization of extrachromosomal Prl gene sequences in drug-treated cells, equal amounts of DNA isolated from Hirt's supernatant and pellet were analyzed by band centrifugation in CsCl, and these results are presented in Figure 6. DNA from Hirt's pellet of control (panel A) and that of drug-treated cells (panel C) sedimented very rapidly, whereas DNA from Hirt's supernatant of control (panel B) and drug-treated cells (panel D) sedimented slower than adenovirus DNA (as indicated by the arrow). From the banding pattern of the two DNA preparations, it seems that the rapidly sedimenting Hirt's pellet DNA is separable from the DNA isolated from Hirt's supernatant. Dot hybridization analysis of the fractionated DNA samples with a [^{32}P]$_pDNA_{Prl}$-insert demonstrates that the amplified Prl gene sequences are enriched in the Hirt's supernatant DNA of drug-treated cells. The highest level of

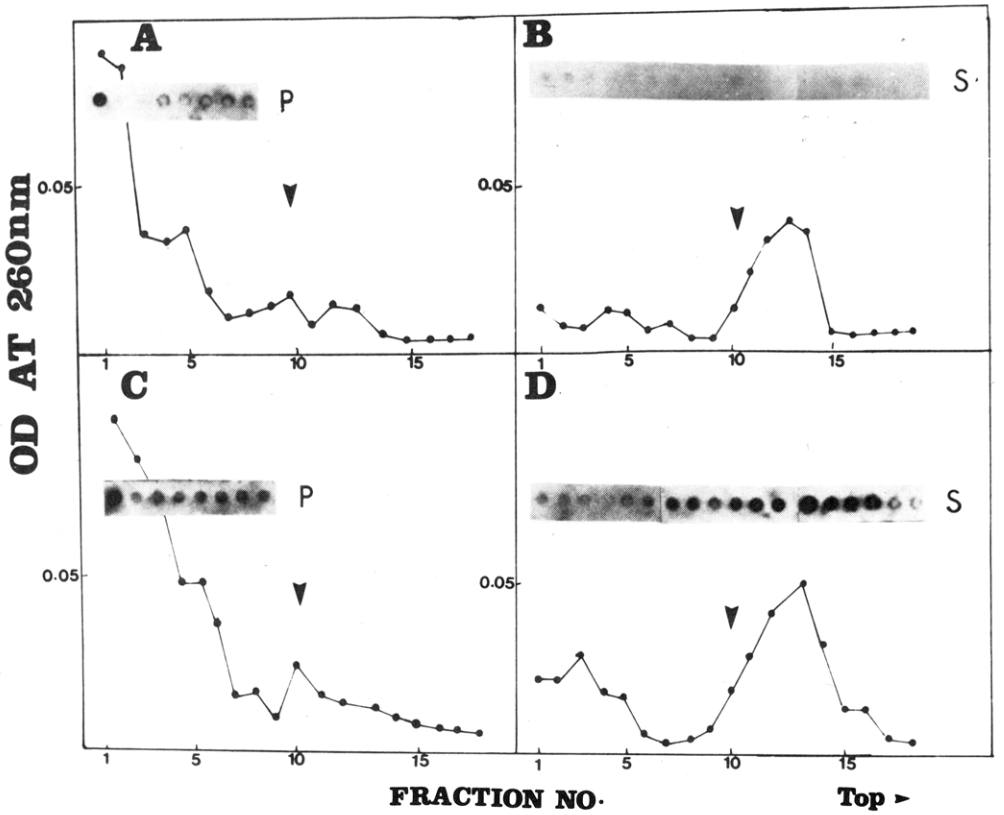

FIGURE 6: Sedimentation velocity analysis of Hirt's pellet and supernatant DNA by band centrifugation in CsCl and measurement of concentrations of Prl gene sequences in fractionated samples. DNA from Hirt's pellet (10 μg, panel A) and supernatant (5 μg, panel B), isolated from control cells, and the DNA from Hirt's pellet (10 μg, panel C) and supernatant (5 μg, panel D) from brdUrd-treated (300 μg/mL, 5 days) cells were subjected to centrifugation in CsCl (ρ = 1.5 μg cm^3) for 3 h, at 35 000 rpm in an SW50.2 rotor (Beckman Instruments) at 20 °C. After centrifugation, fractions (5 drops each) were collected from the bottom of the tubes. Optical density at 260 nm was measured in an aliquot of each fraction, and another aliquot was denatured and analyzed by dot hybridization. The dot hybridization was carried out as described under Materials and Methods. Autoradiographic spots shown in the insets represent the signals generated by DNA from pellet (P) and supernatant (S) of control cells (panels A and B) and that from brdUrd-treated cells (panels C and D). The level of Prl gene sequence in each fraction was measured by hybridization with [^{32}P]$_p$DNA$_{Prl}$-insert (2 × 10^6 cpm/5 mL of hybridization mixture, sp act. 8 × 10^7 cpm/μg of DNA) for 48 h, and autoradiography was for 7 days at −80 °C. The fraction, indicated by the arrow, shows the position of the peak of optical density (at 260 nm) of adenovirus DNA analyzed under identical conditions.

Prl gene sequences can be correlated with the fraction containing the highest concentration of Hirt's supernatant DNA from drug-treated cells. No significant amount of Prl gene sequences can be detected in the Hirt's supernatant DNA prepared from untreated $F_1BGH_12C_1$ cells, whereas the concentration of Prl gene sequences in the Hirt's pellet DNA fractions of drug-treated cells (panel C) is slightly higher than that isolated from control cells (panel A). The increased level of Prl gene sequences is more evident in the Hirt's supernatant DNA fraction of drug-treated cells. These results thus further substantiate the notion that the low molecular weight supernatant DNA is not a shearing product of a high molecular weight pellet DNA. These three lines of evidence, generated by analysis of Hirt's supernatant DNA by (1) dot hybridization, (2) Southern blot analysis, and (3) band centrifugation in CsCl, demonstrate that the drug-induced amplification of the Prl gene is associated with the extrachromosomal DNA.

Discussion

In this paper, the phenomenon of brdUrd-induced amplification of the Prl gene in the GH subclone $F_1BGH_12C_1$ has been further characterized. Several specific questions are posed, the answers to which, we believe, have provided clues to the better understanding of the mechanism of the process of gene amplification in eukaryotic cells. Amplification of specific genes leading to the overproduction of specific proteins in eukaryotic systems have been observed during cellular developmental processes (Brown & Dawid, 1968; Yao et al., 1979; Spradling & Mahowald, 1980). Prl synthesis by pituitary cells is a differentiated function, and Prl production increases during specific physiological processes such as in pregnancy and during lactation. During these periods, Prl is essential for the growth and development of mammary glands, and Prl also stimulates the synthesis of milk protein, casein. Under physiological conditions, stimulation of Prl synthesis is induced by other hormones such as the ovarian steroid, estradiol, and by the hypothalamic tripeptide, TRH. Hormonal regulation of Prl synthesis in the rat pituitary gland and in cultured rat pituitary tumor cells has been studied by several investigators. Such stimulation of Prl synthesis in rat pituitary glands (Maurer et al., 1980) and in GH cells (Potter et al., 1981; Biswas et al., 1982) can be correlated with the increased levels of nuclear and cytoplasmic mRNA$_{Prl}$ sequences. However, the possible role of Prl gene amplification in the process of hormonally stimulated synthesis of Prl in either of these systems was not addressed in those reports. Results presented in this investigation (Figure 1) suggest that stimulation of Prl synthesis in rat pituitary glands during pregnancy and lactation is not associated with Prl gene amplification. Our previous results (Biswas & Hanes, 1982) and results presented in Figure 1 (bottom, panel B, lane B) suggest that in the amplified state the cells have about 40-fold more Prl copies per haploid genome. Evans & Rosenfeld (1979) reported about one to two copies of Prl gene per haploid genome of GH_3 cells. Since we have demonstrated that all GH subclones, including $F_1BGH_12C_1$, have approximately the same number

of Prl gene copies at the basal state, it may be assumed that at the amplified state, $F_1BGH_12C_1$ cells have about 40–80 copies of Prl gene sequences per haploid genome. This conclusion is drawn from the Southern blot analysis of control and drug-treated cell DNA.

Results presented in Figure 2 (row C, dots 1–3) demonstrate that TRH-induced stimulation of Prl synthesis in GH_3 cells is not accompanied by amplification of the Prl gene. The mechanism of stimulation of Prl synthesis in response to hormones in intact rat pituitary gland and in cultured rat pituitary tumor cells is therefore not mediated via gene amplification but via stimulation of transcription. These results also suggest that the observed brdUrd-induced amplification of the Prl gene in $F_1BGH_12C_1$ cells is not a reflection of a cellular process that occurs during the induction of a differentiated function.

Specific gene amplification in eukaryotic cells in response to drugs has also been reported (Schimke et al., 1979). In these instances, the overproduction of a specific gene product such as dihydrofolate reductase in response to the drug methotrexate is directly linked to the mechanism by which the cells acquired the drug-resistant phenotype. Several brdUrd-resistant and -sensitive GH cell strains have been used in this investigation to explore the possible correlation between the drug-resistant phenotype and Prl gene amplification. The primary goal of these experiments has been to determine the levels of Prl gene sequences in each of these strains before and after drug treatment. The levels of Prl gene sequences in each of these GH cell strains do not seem to be significantly different from each other (Biswas & Hanes, 1982). The drug concentrations chosen to study the Prl gene amplification are based on our previous observations that $brdUrd^r$ strains of $F_1BGH_12C_1$ can tolerate as high as 300 μg/mL of the drug and that the same amount of Prl is synthesized by these cells following drug treatment at 300 μg/mL for 5 days or at 30 μg/mL for 10 days (Biswas et al., 1977, 1979).

The proliferation of $brdUrd^r$ cell strains is dependent neither on hormone synthesis nor on the presence of the drug. Results presented in Figure 2 demonstrate that Prl gene amplification is not only observed in the drug-resistant cells (row A, spots 1–6) but also noticed in the drug-sensitive cells following treatment with sublethal concentrations of the drug (row B, spots 8 and 9). This is also accompanied by the increased production of Prl from nondetectable levels to detectable amounts in these cell lines (Biswas et al., 1977). In contrast, in another brdUrd-resistant GH subclone, $F_2BGH_12C_1$, which does not synthesize Prl either in the presence or in absence of the drug, no drug-induced alteration in the level of Prl gene sequences can be detected, whereas, under the same drug treatment conditions, both Prl gene amplification and Prl synthesis can be observed in $F_1BGH_12C_1$ cells. These results suggest that brdUrd-induced Prl gene amplification in GH subclones can be correlated with the mechanism that permits expression of the suppressed gene and is not linked to the drug-resistant phenotype of the cell strains.

brdUrd-induced amplification of the Prl gene is an unstable phenomenon and seemed to be closely related to a drug-induced extrachromsomal process. Characterization of DNA isolated from Hirt's supernatant reveals that neither Gh gene sequences nor albumin gene sequences could be detected in this low molecular DNA preparation. The levels of either of these two genes, i.e., Gh and Alb, in total-cell DNA are not increased following drug treatment and thus suggest that these two genes are not amplified. However, the possibility of amplification of any other gene or genes, other than Prl following brdUrd treatment of these cells, has not been ruled out. Random shearing of chromosomal DNA during preparation of Hirt's supernatant should yield random genomic DNA sequences rather than consistently shearing specifically the amplified Prl gene sequences. The excision of DNA sequences from the chromosomal fragment as an extrachromosomal element is not only size specific but also site specific. The current state of our knowledge does not permit us to speculate on the nature of such an extrachromosomal cellular event.

The mechanisms of differential expression of the Prl gene in several GH subclones is not clearly understood yet. The Prl gene in GH_12C_1 and its derivative $F_1BGH_12C_1$ appears to be suppressed by a specific cellular regulatory mechanism. Results presented here suggest that the release of such suppression of the Prl gene is accompanied by gene amplification. This would suggest that the mechanism of brdUrd-induced gene expression is related to the regulatory mechanism(s) that led to the amplification of the suppressed Prl gene in these cells and is mediated via an extrachromosomal event.

Acknowledgments

We are thankful to Dr. Richard A. Maurer of Iowa State University for providing us with the $cDNA_{Prl}$ recombinant clone, to Dr. G. Page of Massachusetts General Hospital for providing us with the $cDNA_{Gh}$ recombinant clone, and to S. Tilghman of the Institute for Cancer Research, Philadelphia, for providing us with the $cDNA_{Alb}$ recombinant clone. We are also thankful to Dr. Peter Brown of Stanford University, to Dr. J. S. Miller and David Porter of the Department of Biological Chemistry, and to Dr. Thomas Benjamin of the Pathology Department, Harvard Medical School, for their help and advice.

Registry No. 5-Bromodeoxyuridine, 59-14-3; prolactin, 9002-62-4.

References

Biswas, D. K., & Hanes, S. D. (1982) *Nucleic Acids Res. 10*, 3992–4008.

Biswas, D. K., Lyons, J., & Tashjian, A. H., Jr. (1977) *Cell (Cambridge, Mass.) 11*, 431–439.

Biswas, D. K., Abdullah, K. T., & Brennessel, B. A. (1979) *J. Cell Biol. 81*, 1–9.

Biswas, D. K., Hanes, S. D., & Brennessel, B. A. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 66–70.

Brennand, J., Chinault, C. A., Konecki, D. S., Melton, D. W., & Caskey, C. T. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 1950–1954.

Brennessel, B. A., & Biswas, D. K. (1980) *J. Cell Biol. 87*, 6–13.

Brown, D. D., & Dawid, I. B. (1968) *Science (Washington, D.C.) 160*, 272–280.

Dannies, P. S., & Tashjian, A. H., Jr. (1973) *J. Biol. Chem. 248*, 6174–6179.

Evans, G. A., & Rosenfeld, M. G. (1979) *J. Biol. Chem. 254*, 8023–8030.

Evans, G. A., David, D. N., & Rosenfeld, M. G. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 1294–1298.

Gorin, M. B., & Tilghman, S. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1351–1355.

Gross-Bellard, M., Oudet, P., & Chambon, P. (1973) *Eur. J. Biochem. 36*, 32–38.

Gubbins, E. J., Maurer, R. A., Hartley, J. L., & Donelson, J. E. (1979) *Nucleic Acids Res. 6*, 915–930.

Hinegardner, R. T. (1971) *Annu. Rev. Biochem. 39*, 197–201.

Hirt, B. (1967) *J. Mol. Biol. 26*, 365–369.

Kafatos, F. C., Jones, C. W., & Efstratidis, A. (1979) *Nucleic Acids Res. 6*, 1541–1552.

Maurer, R. A., Stone, R., & Gorski, J. J. (1976) *J. Biol. Chem. 251*, 2801–2807.
Maurer, R. A., Gubbins, E. J., Christopher, R. E., & Donelson, J. E. (1980) *J. Biol. Chem. 255*, 2243–2246.
Mayo, K. E., & Palmiter, R. D. (1982) in *Gene Amplification* (Schimke, R. T., Ed.) pp 67–73, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Potter, E., Nicolaisen, K., Ong, E. S., Evans, R. M., & Rosenfeld, M. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6662–6666.
Schimke, R. T., Alt, F. W., Kellems, R. E., Kaufman, R., & Bertino, J. R. (1979) *Cold Spring Harbor Symp. Quant. Biol. 42*, 649–657.
Seeburg, P. H., Shine, J., Martial, J. A., Baxter, J. D., & Goodman, H. M. (1977) *Nature (London) 270*, 486–494.
Southern, E. M. (1975) *J. Mol. Biol. 98*, 503–517.
Spradling, A. C., & Mahowald, A. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 11*, 1096–2002.
Tashjian, A. H., Jr., Bancroft, F. C., & Levine, L. (1970) *J. Cell Biol. 47*, 61–70.
Tlsty, T., Brown, P. C., Johnston, R., & Schimke, R. T. (1982) in *Gene Amplification* (Schimke, R. T., Ed.) pp 231–238, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Vinograd, J., Bruner, R., Kent, R., & Weigle, J. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 902–910.
Wahl, G. M., Padgett, R. A., & Stark, G. R. (1979) *J. Biol. Chem. 254*, 8679–8689.
Yao, M. C., Blackburn, G., & Gall, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 1293–1296.
Zimmer, W. E., Jr., & Schwartz, R. J. (1982) in *Gene Amplification* (Schimke, R. T., Ed.) pp 137–146, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Translational Recognition of Messenger Ribonucleic Acid Caps as a Function of pH†

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ABSTRACT: The degree to which cell-free translation of eukaryotic mRNA is stimulated by the presence of a 5′-terminal 7-methylguanosine-containing cap is affected by a variety of factors including ionic strength, temperature, mRNA concentration, and the type of mRNA. In this report, we show that pH also affects cap dependence. Translation of globin mRNA from which the cap had been enzymatically removed was relatively insensitive to pH compared with capped mRNA. Also, at low pH (6.6–7.2), the cap analogue m^7GTP caused little inhibition of globin mRNA translation in a cell-free system whereas at higher pH the degree of inhibition increased. Finally, the overall extent to which globin mRNA translation could be inhibited at saturating concentrations of m^7GTP increased with increasing pH. It is also shown that the pK_a of the N-1 proton of m^7GTP is affected by mono- and divalent cations. At the K^+ and Mg^{2+} concentrations optimal for cell-free translation, the pK_a is approximately 7.4. Since the pH optimum for translation is near 7.6, both keto and enolate forms of m^7G are present in appreciable amounts. One interpretation for the observed change in cap dependence with pH is that only the enolate form of m^7G is recognized by the cap-binding protein.

Eukaryotic messenger RNA differs from its prokaryotic counterpart in that it contains a 7-methylguanosine moiety linked 5′ to 5′ to the first coded nucleoside (Rottman et al., 1974; Furuichi & Miura, 1975; Adams & Cory, 1975). This structure, referred to as a "cap", is not an obligatory requirement for translation but does accelerate the rate of initiation 5–10-fold [for reviews, see Skatkin (1976) and Banerjee (1980)]. This effect is mediated through interaction of the 5′-terminus of mRNA with a cap-binding protein, presumably the entry point for mRNA into the initiation process (Sonenberg & Shatkin, 1977; Trachsel et al., 1980; Hellmann et al., 1982). The dependence of translation on the cap is strongly affected by ionic strength: at 50 mM potassium acetate (KOAc),[1] cap analogues such as m^7GTP fail to inhibit translation, while at 200 mM KOAc, they exert a maximal inhibition (Weber et al., 1977; Kemper & Stolarsky, 1978; Chu & Rhoads, 1978). The dependence of translation on the cap is also affected by temperature (Weber et al., 1978) and by mRNA concentration (Chu & Rhoads, 1980). While information of this type is useful for interpreting cell-free translational data, its greater importance is in gaining insight into the mechanism by which cap-binding protein and other factors recognize the 5′-terminus of mRNA. We describe here another factor which affects cap dependence, the pH of the cell-free system.

Materials and Methods

Cell-Free Translation System. The messenger-dependent rabbit reticulocyte system of Pelham & Jackson (1976) was used. The sources and concentrations of all components have been described previously (Chu & Rhoads, 1980) with the exception that buffer was added as indicated in the figure legends. Several preparations of reticulocyte lysate were used in the course of this study. These were either prepared as previously described (Chu & Rhoads, 1978) or purchased from Clinical Convenience Inc. (Madison, WI), or Hazelton

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[1] Abbreviations: TAP, tobacco acid pyrophosphatase; m^7GpppA, 7-methylguanosine linked 5′ to 5′ by a triphosphate bridge to adenosine; KOAc, potassium acetate; HPLC, high-performance liquid chromatography; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

0006-2960/83/0422-6084$01.50/0