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Malignant Transformation by H-*ras* Results in Aberrant Regulation of Ribonucleotide Reductase Gene Expression by Transforming Growth Factor-β_1

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Abstract Ribonucleotide reductase is a key rate-limiting and regulatory step in DNA synthesis and plays a crucial role in the coordination of DNA synthesis, DNA repair, and cell proliferation. The present study demonstrates a link between alterations in TGF-β_1 regulation during malignant conversion and the expression of ribonucleotide reductase. H-*ras*-transformed mouse 10T½ cell lines exhibiting malignant potential were examined for possible TGF-β_1-mediated alterations in ribonucleotide reductase expression. Selective induction of ribonucleotide reductase gene expression occurred, since only H-*ras*-transformed highly metastatic cells exhibited marked elevations in ribonucleotide reductase expression, whereas nontransformed normal 10T½ cells were unaffected by TGF-β_1 treatment. These changes occurred without any detectable modifications in DNA synthesis rates, suggesting that these changes were regulated by a novel mechanism independent of the S-phase of the cell cycle. Furthermore, this TGF-β_1-mediated regulation of ribonucleotide reductase expression was shown to occur through an autocrine mechanism. TGF-β_1-modulated regulation of ribonucleotide reductase expression requires de novo protein synthesis and involves, at least in part, transcriptional and post-transcriptional events. Furthermore, evidence is presented to suggest a possible role for protein kinase C-mediated events, protein phosphatases, and G-protein-coupled events in the TGF-β_1-mediated regulation of ribonucleotide reductase expression in H-*ras*-transformed malignant cells. TGF-β_1 regulation of ribonucleotide reductase in highly malignant cells appears to be complex and multifaceted and constitutes an integral part of an altered growth regulatory program.

Key words: TGF-β_1, ribonucleotide reductase, metastasis, aberrant regulation

During malignant progression, numerous and varied biological and biochemical changes occur [Nicolson, 1984; Weber, 1983]. These alterations appear to be controlled through modifications in a discrete set of fundamental regulatory genes, especially oncogenes [Greenberg et al., 1989; Wright et al., 1990a; Egan et al., 1987a,b], which appear to be critically important in cellular functions [Wright et al., 1990b, 1993; Greenberg et al., 1989]. These functions are often subject to regulation by growth factors [Weinberg, 1989; Wright et al., 1993].

Transforming growth factor-β (TGF-β) represents a family of 25-kd homodimeric proteins required for the growth, development, and differentiation of many types of cells [Sporn and Roberts, 1990; Jakowlew et al., 1988; Wakefield et al., 1987; Wilder and Rizzo, 1991]. Although most normal cells are growth inhibited by TGF-β_1, its effects are dependent on many growth conditions [Roberts et al., 1988; Schwarz et al., 1988]. Previously, we have reported that while TGF-β_1 inhibited DNA synthesis in the 10T½ pulmonary fibroblast cell line, H-*ras*-transformed 10T½ cell lines that demonstrated a highly malignant phenotype exhibited stimulation of DNA synthesis following exposure to TGF-β_1 [Schwarz et al., 1988]. Furthermore, we have suggested that this altered response to TGF-β_1 following malignant transformation by *ras*, combined with an elevated rate of secretion of activated TGF-β_1 by these cells [Schwarz et al., 1990], indicates a role for TGF-β in the autocrine stimulation of cell proliferation in malignancy. In the presence of TGF-β_1, metastatic cell populations will exhibit alterations in expres-

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sion of genes involved in the coordination of DNA synthesis and cell proliferation. Previously, we tested this idea by investigating the highly regulated expression of the two genes that code for a rate-limiting enzyme of DNA synthesis, ribonucleotide reductase [Hurta et al., 1991].

Ribonucleotide reductase is responsible for the de novo conversion of ribonucleotides to deoxyribonucleotides essential for DNA synthesis [Wright, 1989; Wright et al., 1990a]. In mammalian cells, ribonucleotide reductase is composed of two dissimilar components often called R1 and R2. Protein R1 is a dimer with a molecular weight of 170,000 and possesses complex substrate and effector-binding sites [Wright et al., 1990a; Thelander et al., 1980]. Protein R2 also exists as a dimer, with a molecular weight of 88,000 and contains non-heme iron and a unique tyrosyl-free radical required for activity [Wright et al., 1990a; McClarty et al., 1987]. The activity of ribonucleotide reductase correlates more closely with DNA synthesis than any other biosynthetic activity [Weber, 1983]. Indeed, in actively growing cells, S-phase appears to be dependent on synthesis of the R2 component, which is rate-limiting for ribonucleotide reductase activity [Wright et al., 1990a; Bjorklund et al., 1990]. Changes in ribonucleotide reductase expression can significantly influence the biological properties of cells [Wright, 1989]. The enzyme may be involved in certain immunodeficiency diseases in humans [Ullman et al., 1979] and has been defined as a mutator locus in mammalian cells [Weinberg et al., 1981]; studies have suggested that the activity is tumor progression linked [Weber, 1983]. In addition, this enzyme is important in DNA repair [Hurta and Wright, 1992b] and in the critical early events in mechanisms of tumor promotion [Hurta and Wright, 1992a; Amara et al., 1994; Chen et al., 1993]. Previous investigations have suggested a link between TGF-β_1 regulation of cellular transformation and alterations in ribonucleotide reductase gene expression [Hurta et al., 1991; Amara et al., 1993]. The present study extends these earlier observations and provides further support of a key role for TGF-β_1 in the aberrant regulation of ribonucleotide reductase in highly malignant H-*ras*-transformed fibrosarcomas.

MATERIALS AND METHODS

Cell Lines and Growth Conditions

Mouse cell lines were routinely cultured at 37°C on plastic tissue culture plates (Lux Scientific) in α-minimal essential medium (α-MEM) (Flow Laboratories), supplemented with antibiotics and 7% (v/v) fetal bovine serum (FBS) (Hyclone Laboratories) as previously described [Hurta et al., 1991]. Cells grew with doubling times of approximately 18–20 h. In experiments using the growth factor TGF-β_1 (R&D Systems, Inc.), a serum-free medium was used that contained 4 μg/ml of transferrin (Sigma Chemical Co.) and 2 μg/ml of insulin (Sigma Chemical Co.) in 100 ml of α-MEM [Hurta et al., 1991; Schwarz et al., 1988]. In this medium, the cells grew with doubling times of approximately 20–22 h. Cells were grown overnight in α-MEM with 7% FBS, then switched to the serum-free medium for 18 h prior to exposure to 10 ng/ml TGF-β_1 at predetermined times [Hurta et al., 1991]. Cells were removed from the surface of tissue culture plates using a 0.3% buffered trypsin solution (Difco Laboratories) [Hurta and Wright, 1990].

Northern Blot Analysis

Total cellular RNA was prepared by a rapid extraction method [Gough, 1988] and subjected to electrophoresis through 1% formaldehyde–agarose gels, followed by transfer to Nytran nylon membranes (Schleicher and Schuell). Blots were prehybridized and hybridized as we have outlined previously [McClarty et al., 1990]. Hybridization occurred either in the presence of a ^{32}P-labeled *Nco*I-generated fragment containing the cDNA of clone 65 (R1) or the *Pst*1-generated fragment containing the cDNA of clone 10 (R2) [McClarty et al., 1987] or in the presence of a ^{32}P-labeled *Bgl*II-generated TGF-β_1 fragment from the pPK9A plasmid [Samuel et al., 1992], or in the presence of a ^{32}P-labeled *Eco*R1-generated fragment of *jun*B obtained from the p456.20 plasmid (provided by D. Nathans, John Hopkins University, Baltimore, MD). Glyceradihyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to determine RNA loading. Probes were labeled using an oligolabeling kit (Pharmacia LKB Biotechnology).

Ribonucleotide Reductase Assay

CDP reductase activity was determined by the method of Steeper and Stuart [1970], as modified by Cory and Whitford [1972], using [^{14}C]-CDP (Moravek Biochemicals, Inc.) as substrate and *Crotalus atrox* snake venom to hydrolyze the nucleotides, as we have previously described

[Hards and Wright, 1981; Lewis et al., 1978]. The CDP reduction reaction mixture contained in a final volume of 150 μl, [^{14}C]-CDP (0.05 μCi, 7.5 nmol), dithiothreitol (900 nmol), magnesium acetate (600 nmol), ATP (300 nmol), and a quantity of enzyme preparation. Reactions were initiated by the addition of enzyme and then carried out for 20 min at 37°C for CDP reduction and 1 h at 37°C in the presence of snake venom. All reactions were terminated by boiling for 5 min; then 0.5 ml of distilled water was added to each assay tube, and heat-precipitable material was removed by centrifugation. The supernatant was retained and the deoxycytidine was separated from the cytidine compounds on 5 × 80-mm Dowex-1-borate columns (BioRad Laboratory). Briefly, the separation depends on the formation of a complex between ribonucleosides and borate ions in the column resin. Deoxyribonucleosides do not possess a cis-diol, and therefore cannot form a complex. Deoxycytidine was eluted from the column with 5 ml of distilled water; 7 ml of Scintiverse (Fisher Scientific) was then added. Radioactivity was determined in a Beckman model LS7800 liquid scintillation spectrophotometer.

DNA Synthesis Assay

DNA synthesis in cells cultured in 6-well Nunclon plates (Nunc) was measured by incorporation of [^{3}H]thymidine into 10% trichloroacetic acid (TCA)-insoluble material [Hurta et al., 1991]. C3 cells [Egan et al., 1987a] were grown for 24 h in α-MEM with 7% FBS, then washed three times with phosphate-buffered saline (PBS), followed by exposure to serum-free medium in the presence or absence of TGF-β_1 (10 ng/ml) for various periods of time; they were then pulsed with 50 μCi/ml CH_3-[^{3}H]thymidine (ICN Radiochemicals) for 2 h [Hurta et al., 1991]. Medium was then removed, and a 0.3% buffered trypsin solution was added to the wells for 30 min at 37°C. Ice-cold TCA was added to a final concentration of 10% for 30 min at 4°C. Cellular material was passed through 2.4-cm glass microfiber filters (Whatman) prewashed with 10% ice-cold TCA. Wells were washed twice with ice-cold 10% TCA; these washes were applied to the filters. Filters were then washed with 95% ethanol, air-dried, and placed into 8 ml of Aquasol-2 (DuPont, New England Nuclear). Radioactivity was determined by liquid scintillation spectroscopy using a model LS7800 scintillation counter (Beckman).

RESULTS

Properties of T-24 H-*ras*-Transfected 10T½ Mouse Fibroblasts

Isolation and characterization of the cell lines used in this study have been discussed previously [Egan et al., 1987a; Schwarz et al., 1988, 1990]. Briefly, 10T½ mouse fibroblast cells were transfected with the plasmid pAL8A, which contains T-24 H-*ras* and the neo^R gene. After transfection, cell lines were isolated and characterized. A morphologically transformed cell line, C3 was used here for further study. A summary of the biological properties of these cell lines is presented in Table I. The 10T½ parental cell line is not tumorigenic, whereas the C1 and C3 cell lines exhibit increasing malignant characteristics, with C3 highly malignant. A cell line called clone 17.18 was isolated from the C1 cell line as described previously [Samuel et al., 1992]. In brief, to obtain this cell line, C1 cells (Table I) were co-transfected by lipofection [Felgner et al., 1987] with the pPK9A plasmid [Samuel et al., 1992], containing the coding region of TGF-β_1 under the control of a zinc-sensitive metallothionein promoter and the hygromycin resistance (PY3) marker gene [Samuel et al., 1992]. The pPK9A plasmid contains the coding region of the porcine TGF-β_1 cDNA inserted between the metallothionein promoter and the human growth hormone (hGH) polyadenylation sequence [Samuel et al., 1992]. Normally, TGF-β_1 is secreted in a latent form [Gentry et al., 1988]. However, in order to ensure secretion of active TGF-β_1, serines were substituted for cysteines at 223 and 225 in the propeptide. This mutation results in the release of bioactive TGF-β_1 [Brunner et al., 1989]. A clone designated 17.18 showed very low basal level of TGF-β_1 mRNA, which was only slightly higher in the uninduced cells than in the nontransfected C1 cell line [Samuel et al., 1992]. This clone, 17.18, was then used for further investigations.

Selective Induction of Ribonucleotide Reductase Gene Expression by TGF-β_1

The effects of TGF-β_1 on ribonucleotide reductase gene expression in 10T½ and C3 cells shown in Table I are presented in Figure 1. Elevations of R2 and R1 mRNA levels were observed following TGF-β_1 treatment, but this occurred only in the highly malignant cell line, C3. No increase in either R2 or R1 gene expression was detected in TGF-β_1-treated parental 10T½ cells, whereas

TABLE I. Tumorigenic and Metastatic Properties of Mouse 10T½ and C3 Cell Lines*

Cell line	Tumorigenicity frequency[a]	Experimental metastases[b]		Degree of malignancy
		Frequency	No. of lung nodules (mean ±SE)	
10T½	0/12	0/12	0	Normal
C1	13/13	20/27	14 ± 5	Intermediate
C3	11/11	14/14	121 ± 20	High

*Data summarized from previously reported results [Egan et al., 1987a].
[a]Tumorigenicity was determined following subcutaneous injections of 3×10^5 cells.
[b]Injections were performed with 3×10^5 cells.

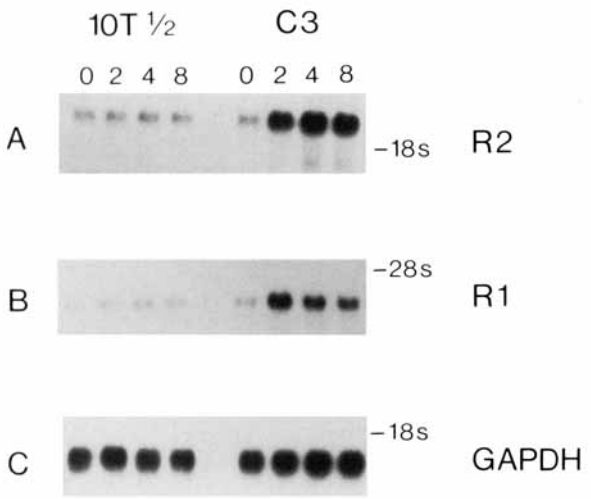

Fig. 1. Ribonucleotide reductase gene expression following treatment with TGF-β_1 in 10T½ **(left)** and C3 **(right)** cells. Northern blot analyses of R2 mRNA and R1 mRNA levels in the absence of TGF-β_1 and following exposure to TGF-β_1 (10 ng/ml) for 2, 4, and 8 h. The total cellular RNA blots were probed with **(A)** R2, **(B)** R1, and **(C)** GAPDH cDNA, as a control for loading. The position of ribosomal markers is indicated. The autoradiograms were exposed for 24 h at −70°C with intensifying screens.

in malignant C3 cells, 4.2-, 5.7-, and 5.1-fold and 4.8-, 3.3-, and 3.2-fold increases in R2 and R1 gene expression, respectively, were detected following exposure of cells to TGF-β_1 for 2, 4, and 8 h, respectively.

To investigate directly the relationship between the expression of R2 and R1 with TGF-β_1 treatment illustrated above, clone 17.18 was investigated further. A time-course analysis with this clone showed that TGF-β_1 message levels increased 2 h postinduction with $ZnSO_4$ exposure; this expression increased to about 5-fold above untreated controls after 8 h, after which no further elevation was noted (Fig. 2A). Expression of the *jun*B gene was observed after 2-h $ZnSO_4$ treatment of the 17.18 cell line and occurred coincidentally with the elevation of TGF-β_1 mRNA (Fig. 2A). Increased R1 and R2 mRNA levels were also observed 2 h postinduction of the 17.18 cells with $ZnSO_4$ (Fig. 2A). The time-course analysis showed that 5.2- and 5.9-fold elevations in R1 and R2 mRNA levels, respectively, occurred 2 h postinduction with $ZnSO_4$. The increase in R1 mRNA level was maintained over the entire time course (Fig. 2A). R2 mRNA levels remained elevated, with maximum increases detected at 24–48 h postinduction of $ZnSO_4$ of about 11.4-fold.

Since TGF-β_1, *jun*B, and R2 and R1 were induced after 2 h of $ZnSO_4$ treatment, the expression of these genes at earlier times was investigated to determine the order of induction. In this analysis, TGF-β_1 mRNA levels were elevated after 30 min of $ZnSO_4$ treatment, whereas *jun*B mRNA levels were not increased until 45 min postinduction (Fig. 2B). Interestingly, R2 and R1 mRNA levels were elevated after TGF-β_1 mRNA levels were increased but occurred coincident with increases in *jun*B mRNA. R1 and R2 mRNA levels were elevated 3.4- and 5.3-fold respectively, following 45 min $ZnSO_4$ treatment (Fig. 2B).

Ribonucleotide Reduction in Cells Treated With TGF-β_1

Ribonucleotide reductase activity is elevated dramatically during the period of DNA synthesis and correlates more closely with this process than any other biosynthetic activity [Lewis et al., 1978; Weber, 1983]. In actively proliferating cells, S-phase appears to be dependent on the synthesis of the R2 component, which is rate limiting for enzymatic activity [Wright et al., 1990a]. Assays of ribonucleotide reductase activity at various periods of time following TGF-β_1 exposure revealed that ribonucleotide reductase activity was increased in the highly malignant C3 cell line. Ribonucleotide reductase activity

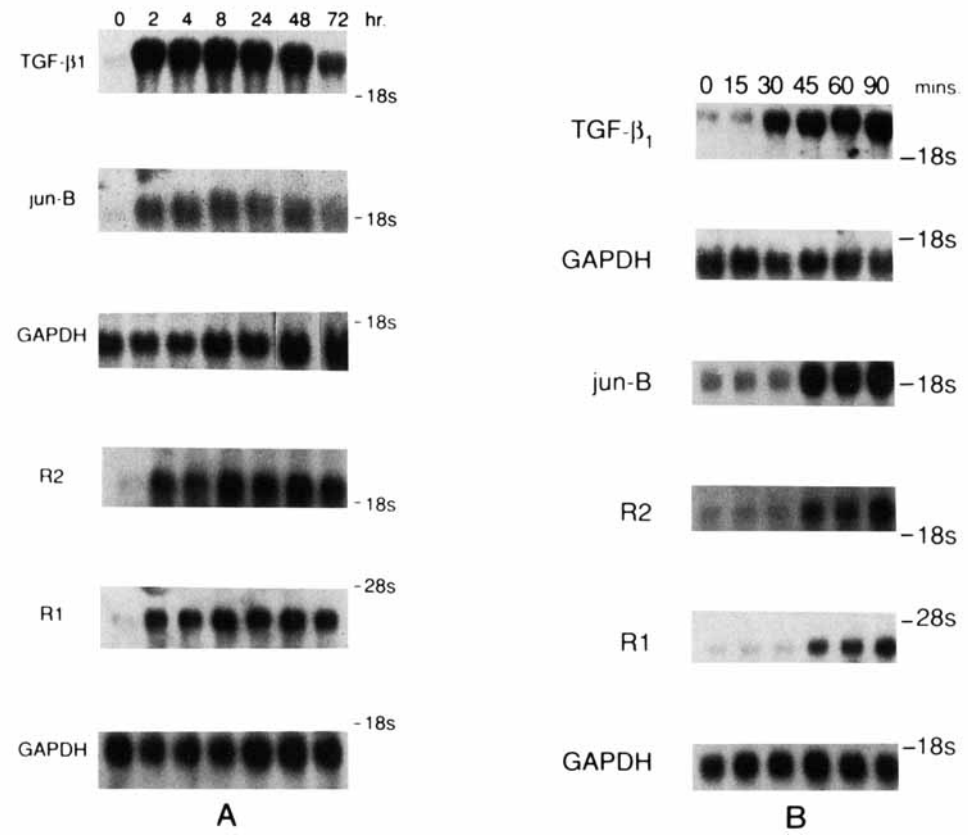

Fig. 2. **A:** Overexpression of TGF-β_1 and R2 and R1 in clone 17.18 following $ZnSO_4$ treatment. Kinetics of induction of TGF-β_1, *jun*B, R2, and R1 mRNA levels are significantly increased by 2 h of incubation. GAPDH loading controls are shown. All blots were exposed for 24 h except the *jun*B blot, which was exposed for 48 h at −70°C with intensifying screens. The TGF-β_1 and *jun*B Northern blot analyses have been carried out previously and are included here for ease of comparison [Hurta et al., 1993]. **B:** TGF-β_1 induction following $ZnSO_4$ treatment of clone 17.18 cells occurs prior to *jun*B induction. TGF-β_1 and *jun*B mRNA levels were elevated at 30 min and 45 min, respectively, postinduction of clone 17.18 cells with $ZnSO_4$. R2 and R1 mRNA levels were both elevated at 45 min postinduction of clone 17.18 cells with $ZnSO_4$ coincident with elevated *jun*B mRNA expression. GAPDH loading controls are also shown. All blots were exposed for 24 h at −70°C with intensifying screens. TGF-β_1 and *jun*B Northern blot analyses have been carried out previously and are included here for ease of comparison [Hurta et al., 1993]. No change in TGF-β_1, *jun*B, R2, or R1 mRNA levels were detected following $ZnSO_4$ treatment of the C1 tumor cell line (data not shown).

increased on average 2–3.5-fold in C3 cells exposed to TGF-β_1. On the contrary, there was no elevation in ribonucleotide reductase enzymatic activity in normal 10T½ cells following exposure to TGF-β_1 (Table II), in keeping with the inhibition of DNA synthesis and cell proliferation usually observed following treatment of normal fibroblasts with TGF-β_1 [Wright et al., 1993].

Induction of Ribonucleotide Reductase in Relation to S-Phase

The changes observed in R1 and R2 gene expression and ribonucleotide reductase enzyme activity observed in C3 cells occurred relatively quickly following exposure to TGF-β_1, making it unlikely that a block at S-phase or an unusual movement of cells into S-phase could account for the marked modifications in ribonucleotide reductase expression. To investigate this possibility directly, incorporation of [CH_3-^{3}H]thymidine into DNA was measured [Hurta et al., 1991]. Figure 3 illustrates that the modifications of ribonucleotide reductase expression by TGF-β_1 treatment occurring in C3 cells took place prior to any detectable change in the rates of DNA synthesis. These findings support the hypothesis that TGF-β_1 is able to induce ribonucleotide reductase gene expression and enzymatic activity in these malignant H-*ras*-transformed cells without altering the proportion of cells in S-phase.

TABLE II. Ribonucleotide Reductase Enzymatic Activity* in 10T½ and C3 Cells Following Exposure to TGF-β_1

Cell line	Exposure to TGF-β_1[a] (h)	Ribonucleotide reductase activity (nM CDP reduced/ h/mg protein)
10T½	0	0.77 ± 0.20
	2	0.70 ± 0.38
	4	0.59 ± 0.05
	8	0.63 ± 0.28
C3	0	2.01 ± 0.54
	2	3.96 ± 0.63
	4	5.42 ± 0.18
	8	7.14 ± 0.10

*Results shown are from duplicate assays.
[a]The concentration of TGF-β_1 was 10 ng/ml. Control cells received only BSA/HCl in which TGF-β_1 was reconstituted and activated.

Effect of TGF-β_1 Treatment on Transcription of R1 and R2 Genes

The possibility that the increases in R2 and R1 message levels observed in malignant cells following TGF-β_1 exposure were due to changes in gene transcription rates was tested by pretreating C3 cells with the transcription blocker, actinomycin D [Hurta and Wright, 1992b] prior to exposure to TGF-β_1. As illustrated in Figure 4, actinomycin D prevented the elevation in both R2 and R1 message levels previously observed following exposure of these cells to TGF-β_1. In the absence of actinomycin D, 5.4- and 7.0-fold increases in R2 mRNA levels and 4.0- and 4.6-fold increases in R1 mRNA levels were evident at 2 and 4 h postexposure to TGF-β_1, respectively. In the presence of the transcription blocker, no increases in either R2 or R1 message were observed following TGF-β_1 treatment. In control experiments, actinomycin D alone did not affect R2 or R1 message levels (data not shown). These findings suggest that the TGF-β_1 effects on ribonucleotide reductase gene expression are mediated, at least in part, by changes in the transcriptional apparatus.

Effect of Protein Synthesis Inhibition on Ribonucleotide Reductase (R2 and R1) mRNA Induction by TGF-β_1

To determine whether the TGF-β_1-mediated elevations in R1 and R2 mRNA required protein synthesis, C3 cells were exposed to TGF-β_1 in the presence or absence of 10 μg/ml cycloheximide, an inhibitor of eukaryotic protein synthesis [Phillips and Crowthers, 1986; Le et al., 1992]. Figure 5 shows that cycloheximide treatment reduced the TGF-β_1-mediated accumulation of R1 and R2 mRNA in C3 cells. In the absence of cycloheximide, 4.5- and 5.2-fold increases in R1 and R2 mRNA levels, respectively, were noted following exposure to TGF-β_1 for 5 h. When cycloheximide was present, only 3.0- and 3.2-fold elevations in R1 and R2 mRNA levels, respectively, were observed (Fig. 5). Cycloheximide alone did not elevate R1 or R2 mRNA levels.

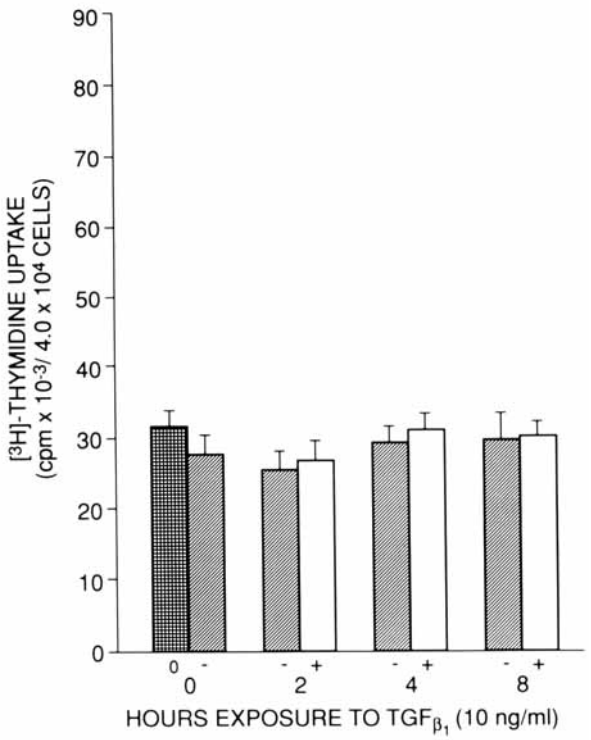

Fig. 3. DNA synthesis in C3 cells treated with TGF-β_1. Cells were stimulated with TGF-β_1, and trichloroacetic acid-insoluble ^{3}H-thymidine incorporation was determined as described under Methods and Materials. The concentration of TGF-β_1 used was 10 ng/ml. −, in the presence of 4 mM HCl in 1 mg/ml bovine serum albumin; +, in the presence of TGF-β_1; 0, no additions.

Stability of Ribonucleotide Reductase Message in Malignant C3 Cells Following Exposure to TGF-β_1

Alterations in the rates of decay of mature message in response to external stimuli are a common mechanism for regulating message levels post-transcriptionally [Raghow, 1987; Chen et al., 1993; Amara et al., 1993, 1994]. TGF-β_1-mediated elevations of R2 and R1 mRNA levels occurred, at least in part, through changes in

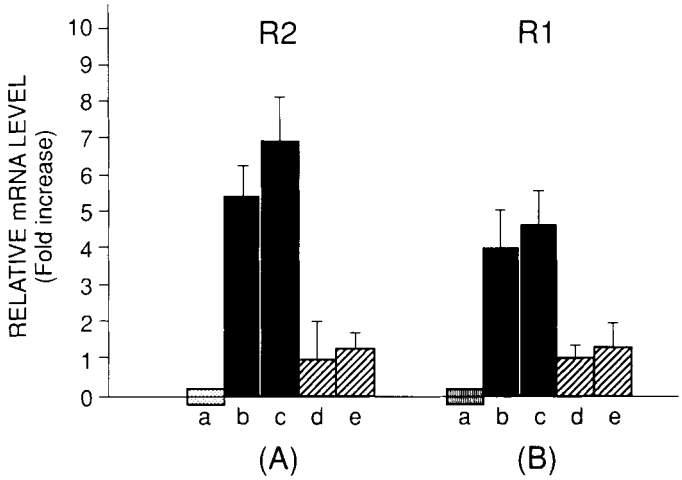

Fig. 4. Actinomycin D prevents the TGF-β_1-induced elevations of R2 and R1 message levels and evaluation of relative R2 and R1 mRNA levels in C3 cells in the absence or the presence of actinomycin D. **A:** Relative R2 mRNA levels in control cells in the absence of actinomycin D and TGF-β_1 (a), R2 mRNA levels in C3 cells in the absence of actinomycin D but with TGF-β_1 for 2 h (b) and 4 h (c); R2 mRNA levels in C3 cells in the presence of actinomycin D and TGF-β_1 for 2 h (d) and 4 h (e). **B:** Relative R1 mRNA levels in C3 cells under the same conditions described above. Actinomycin D concentration was 5 μg/ml, and TGF-β_1 was added at a final concentration of 10 ng/ml. Actinomycin D alone did not affect either R2 or R1 mRNA levels (data not shown).

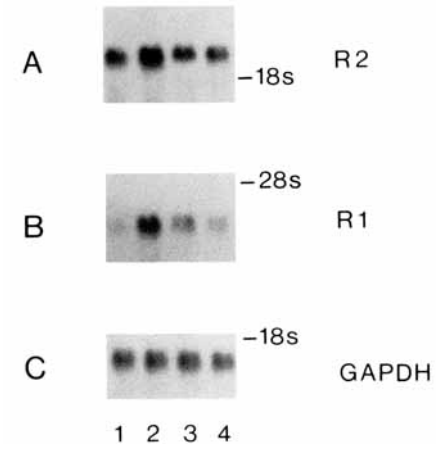

Fig. 5. Cycloheximide treatment decreases the TGF-β_1-induced elevation of R2 and R1 message elevations. **A:** Northern blots of R2 mRNA levels, control C3 cells (1), C3 cells exposed to TGF-β_1 (10 ng/ml) for 5 h (2), cells exposed to TGF-β_1 (10 ng/ml) and cycloheximide (10 μg/ml) for 5 h (3) and C3 cells in the presence of cycloheximide (10 μg/ml) (5-h exposure) alone (4). **B:** Northern blots of R1 mRNA levels as described above are shown. **C:** GAPDH loading controls for the R2 and R1 Northern blots are shown. R2, R1, and GAPDH autoradiograms were exposed for 24, 48, and 24 h, respectively, at −70°C with intensifying screens.

the transcriptional process (see Fig. 4), the possibility that changes at the post-transcriptional level in response to TGF-β_1 can also occur was investigated. The rate of decay of the mature R1 and R2 messages was determined in untreated, and in TGF-β_1-treated, C3 cells after exposure to an inhibitor of transcription initiation, 5,6,dichloro-1-β-D-ribofuranosylbenzimidazole [Tamm and Sehgal, 1978; Mukherjee and Molloy, 1987]. As shown in Figure 6, R2 and R1 mRNA levels in C3 cells are more stable following TGF-β_1 treatment. Assuming that the decay of R2 and R1 message after blocking RNA synthesis follows first-order kinetics, the half-life of R2 and R1 mRNA in TGF-β_1-treated cells, estimated by extrapolation, was approximately increased by 3.6- and 4.3-fold for R2 and R1, respectively, over that found in cells not treated with TGF-β_1. The half-life of the R2 message in untreated cells was estimated to be 2 h, whereas in growth factor-treated cells, the half-life of the R2 message was found to be 7.2 h. The half-life of the R1 message in untreated cells was estimated to be 2.3 h, while in TGF-β_1-treated C3 cells, the half-life of R1 mRNA was estimated to be 10 h. These observations suggest that TGF-β_1 is capable of regulating R2 and R1 mRNA levels through mechanisms of post-transcriptional stabilization.

Possible Role for Protein Kinase C-Mediated Events and Protein Phosphatases in the TGF-β_1-Mediated Regulation of Ribonucleotide Reductase

The involvement of a protein kinase cascade in the action of some mitogens has been proposed [Ralph et al., 1990; Pelech et al., 1990]. A rapid but transient increase in R2 gene expres-

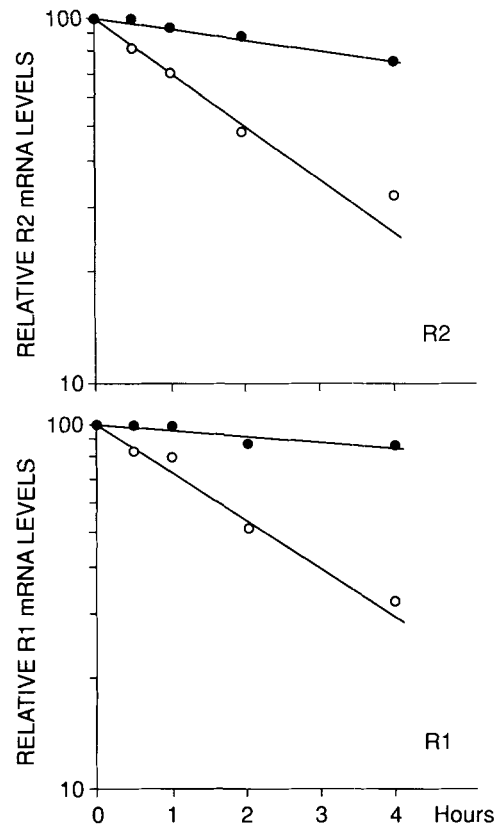

Fig. 6. Stability of R2 and R1 mRNA in untreated and TGF-β_1-treated C3 cells. C3 cells exposed to TGF-β_1 (10 ng/ml) (treated cells) (●), or to BSA/HCl solution (the vehicle in which TGF-β_1 is dissolved/activated) (untreated cells) (○) for 2 h were subsequently treated with 5,6-dichloro-1-β-D-ribofuranosyl benzimidazole (63 μM). Total cellular RNA was isolated at the times indicated and subjected to Northern blot analysis as described. The relative levels of R2 and R1 mRNA were determined by densitometric evaluation of autoradiograms exposed in the linear range for each set of samples. The results presented are from duplicate experiments.

sion in BALB c/3T3 fibroblasts treated with the tumor promoter, 12-0-tetradecanoyl-phorbol-13-acetate (TPA) has been described, and after 24-h TPA treatment, ribonucleotide reductase activity and R2 gene expression returned to approximately the unstimulated condition [Choy et al., 1989]. In C3 cells, TPA treatment causes a rapid but transient elevation of both R2 and R1 gene expression, with R2 and R1 mRNA levels returning to approximately unstimulated levels following 24–48-h TPA treatment (data not shown). Although TPA activates protein kinase C (PKC) [Nishizuka, 1986], the precise pathway responsible for transducing the signal generated through PKC activation is unknown. We hypothesized that the TPA and TGF-β_1 effects on R2 and R1 gene expression in C3 cells may involve some components of the same pathway. To test this hypothesis, C3 cells were pretreated with TPA (0.1 μM) for 48 h prior to exposure to TGF-β_1. Extended treatment with TPA results in a downregulation of PKC activity [Young et al., 1987]. Following this 48-h TPA pretreatment, cells were exposed to TGF-β_1 for 2 and 4 h, respectively. Total cellular RNA was prepared and Northern analysis was performed. In C3 cells, unexposed to TPA, R2 and R1 gene expression was increased 5.5- and 4.4-fold, respectively, following exposure to TGF-β_1 for 4 h (Fig. 7). However, in TPA-pretreated C3 cells, increases in R2 mRNA expression were 1.1- and 1.3-fold following exposure to TGF-β_1 for 2 and 4 h, respectively (Fig. 7). Similarly, in TPA-pretreated C3 cells, only 1.6- and 1.5-fold elevations in R1 mRNA levels were noted in cells exposed to TGF-β_1 for 2 and 4 h, respectively (Fig. 7). These results show that the TGF-β_1-induced elevation of R2 and R1 message levels can be reduced by TPA pretreatment, suggesting that TGF-β_1 may be using, to some extent, a PKC-dependent pathway to modulate ribonucleotide reductase expression in C3 cells.

Previously, we have demonstrated that okadaic acid, a non-phorbol ester tumor promoter, can rapidly elevate R2 and R1 expression in BALB c/3T3 cells, in the short term [Hurta and Wright, 1992b]. Okadaic acid specifically inhibits protein phosphatases 1 and 2A/2B; it has been suggested that prolonged exposure to okadaic acid results in the inhibition of protein phosphatase activity and that this prolonged phosphatase inhibition reduces the tumorigenic properties of cells [Cohen et al., 1990]. To test the possibility that protein phosphatases may also play a role in TGF-β_1 regulation of R2 and R1 expression, C3 cells were pretreated with okadaic acid for 48 h prior to exposure to TGF-β_1 for 2, 4, and 8 h, respectively. Interestingly, in cells pretreated with okadaic acid for 48 h, TGF-β_1 was unable to elevate either R2 or R1 mRNA levels in such cells (Fig. 8). In control cells, that is, cells without okadaic acid pretreatment, but exposed to TGF-β_1 for 8 h, 7.7- and 6.1-fold increases in R2 and R1 mRNA levels were evident, suggesting that the TGF-β_1 was indeed functional in these experiments (Fig. 8). These findings further suggest a possible role for protein phosphatases in the TGF-β_1-mediated modulation of ribonucleotide reductase expression in malignant fibrosarcomas.

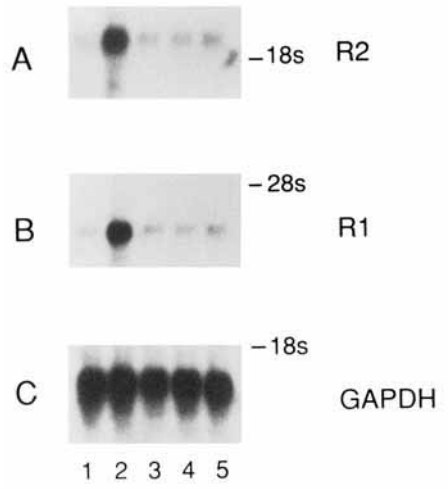

Fig. 7. Possible role of protein kinase C-mediated events in TGF-β_1 regulation of ribonucleotide reductase gene expression. C3 cells were exposed to TPA (0.1 μM) for 48 h prior to exposure of cells to TGF-β_1 (10 ng/ml). Total cellular RNA was isolated, and Northern blot analysis was performed. **A:** Northern blots of R2 mRNA levels are shown in C3 cells not exposed to either TGF-β_1 or TPA (*lane 1*); in C3 cells exposed to TGF-β_1 for 4 h, no exposure to TPA (positive control cells) (*lane 2*); in cells pretreated with TPA for 48 h and then exposed to TGF-β_1 for 2 h (*lane 3*), and for 4 h (*lane 4*); in cells exposed to TPA for 48 h, and no exposure to TGF-β_1 (*lane 5*). Control cells (*lane 1*) received only the vehicle in which the TGF-β_1 and TPA solutions were made. **B:** Northern blots of R1 mRNA levels in C3 cells as described above. **C:** GAPDH mRNA levels as loading controls are shown. R2, R1, and GAPDH autoradiograms were exposed for 24, 24, and 48 h, respectively, at −70°C with intensifying screens.

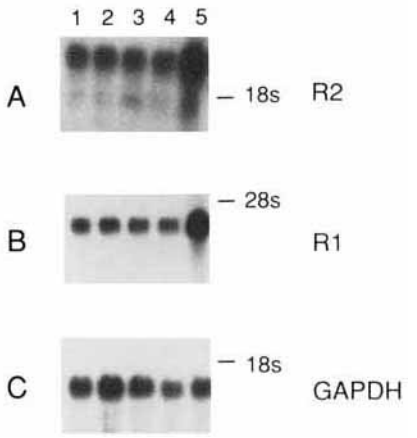

Fig. 8. Possible role for protein phosphatases in TGF-β_1 regulation of ribonucleotide reductase gene expression. C3 cells were pretreated with okadaic acid (10 nM) for 48 h (to downregulate the phosphatases) prior to exposure to TGF-β_1. Total cellular RNA was isolated and Northern blot analysis was performed. **A:** Northern blots of R2 mRNA levels are shown in C3 pretreated with okadaic acid, then exposed to BSA/HCl solution and in the absence of TGF-β_1 (controls cells) (1); in okadaic acid-pretreated cells, following exposure to TGF-β_1 (10 ng/ml) for 2 h (2), for 4 h (3), and for 8 h (4), respectively. R2 mRNA levels are also shown in cells exposed only to TGF-β_1 (10 ng/ml) for 8 h (5). These cells were not exposed to okadaic acid (hence no inactivation of protein phosphatases) prior to treatment with TGF-β_1 (positive control cells). **B:** Northern blots of R1 mRNA levels in C3 cells as described above. **C:** GAPDH mRNA levels as loading controls are shown. R2, R1, and GAPDH autoradiograms were exposed for 48, 48, and 24 h, respectively, at −70°C with intensifying screens.

Possible Involvement of G-Proteins in TGF-β_1-Regulated Ribonucleotide Reductase Gene Expression

Previously, it has been suggested that both G-protein-dependent and -independent signaling may be involved in TGF-β_1-regulated events [Howe et al., 1989]. To test the possibility of G-protein involvement in the TGF-β_1 modulation of R2 and R1 gene expression, C3 cells were pretreated with pertussis toxin alone or in combination with TGF-β_1. Total cellular RNA was isolated, and R2 and R1 gene expression was evaluated by Northern blot analysis. Treatment of C3 cells with TGF-β_1 for 2 h resulted in 4.7- and 5.8-fold elevations of R1 and R2 mRNA levels, respectively (Fig. 9). Treatment of C3 cells with pertussis toxin alone did not alter the level of R1 and R2 mRNA expression. However, TGF-β_1-induced elevations of R1 and R2 gene expression were markedly reduced in cells exposed to pertussis toxin. Only 2.3- and 2.6-fold elevations of R1 and R2 mRNA levels, respectively, were noted following TGF-β_1 exposure to cells treated with pertussis toxin. This observation suggests a possible role(s) for a pertussis toxin-sensitive G-protein(s) involvement in the TGF-β_1-modulated upregulation of ribonucleotide reductase gene expression.

DISCUSSION

Obvious alterations in the expression of R2 and R1 gene expressions were observed in response to TGF-β_1 exposure in highly malignant H-*ras*-transformed C3 cells. Normal 10T½ cells did not exhibit these changes. These observations are consistent with and substantiate previous studies of TGF-β_1 regulation of the ribonucleotide reductase genes and DNA synthesis in normal and malignant cells [Hurta et al., 1991], which showed that malignant *ras*-transformed cells contain alterations in signal transduction pathways that lead to novel regulation of this growth-related activity.

The present investigation also demonstrated directly the relationship between TGF-β_1 expres-

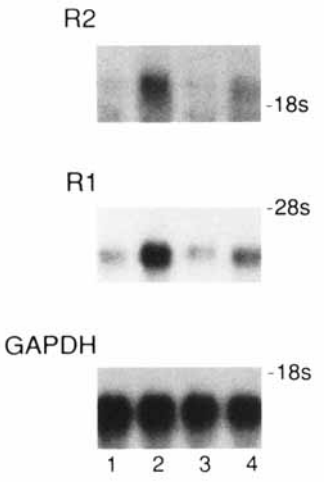

Fig. 9. Possible involvement of G-protein signaling events in TGF-β_1 regulation of ribonucleotide reductase gene expression. Northern blot analysis of R2 mRNA in C3 cells is presented. (1) C3 control cells; (2) C3 cells exposed to TGF-β_1 (10 ng/ml) for 2 h; (3) C3 cells treated with pertussis toxin (50 ng/ml) for 2 h; (4) C3 cells exposed to pertussin toxin (50 ng/ml) and to TGF-β_1 for 2 h. Northern blot analysis of R1 mRNA in C3 cells is provided as described above. GAPDH mRNA levels as a loading control are also presented. The R2, R1, and GAPDH autoradiograms were exposed at −70°C with intensifying screens for 24, 24, and 8 h, respectively.

sion and enhanced R2 and R1 gene expression, using a metallothionein-inducible TGF-β_1-expressing clone, designated 17.18. Secretion of TGF-β_1 in clone 17.18 closely follows TGF-β_1 mRNA levels, indicating that the mutated gene is regulated by stimulation of the metallothionein promoter [Samuel et al., 1992]. Paralleling the increases in TGF-β_1 gene expression, corresponding elevations in R2, R1, and *jun*B mRNAs were observed. Notably, *jun*B expression is an early genomic response in cells sensitive to TGF-β_1 stimulation [Pertovaara et al., 1989]. The rapid *jun*B and R2 and R1 response in clone 17.18 following exposure to $ZnSO_4$ immediately after TGF-β_1 stimulation suggests that the induction is probably occurring through the autocrine action of the secreted bioactive TGF-β_1 homodimer. It should be noted that the effect of TGF-β_1 on clone 17.18, and consequently on increased R2 and R1 gene expression, may also be occurring through an intracrine pathway. Whether this pathway plays a role, and to what extent, is not readily apparent.

S-phase-specific expression of R1 and R2 mRNAs has been reported [Bjorklund et al., 1990]. It is noteworthy that the changes in R1 and R2 gene expression following TGF-β_1 exposure occurred prior to any detectable changes in the rates of DNA synthesis. These observations are consistent with previous investigations in which TGF-β_1 effects on DNA synthesis in cell lines examined in this present study have been described [Schwarz et al., 1988, 1990]. No stimulation of DNA synthesis in malignant C3 cells was observed until at least 18–24 h of TGF-β_1 treatment (data not shown). These observations support the interesting concept that TGF-β_1 induces ribonucleotide reductase gene expression without changing the proportion of cells in S-phase. This is noteworthy because several previous investigations have indicated ribonucleotide reductase activity normally correlates very closely with the S-phase of the cell cycle [Weber, 1983; McClarty et al., 1986], and models of the regulation of mammalian ribonucleotide reductase show that control can occur through the synthesis and breakdown of the R2 component with relatively little change in protein R1 [Wright et al., 1990a]. Previously, we have suggested that the potential to regulate mammalian ribonucleotide reductase gene expression independently of S-phase exists in normal cells as a means of responding to DNA damage and DNA repair requirements [Hurta and Wright, 1992a]. Studies with tumor promoters [Choy et al., 1989; Hurta and Wright, 1992b; Chen et al., 1993; Amara et al., 1994] also support the contention of a role for regulation of ribonucleotide reductase expression outside of S-phase constraints.

Increased levels of R2 and R1 message expression occurred in malignant cells following TGF-β_1 exposure at least partly due to modifications in the transcriptional efficiency of the R2 and R1 genes. Evidence was presented that changes at the post-transcriptional level in response to TGF-β_1 can also occur in malignant H-*ras*-transformed cells. TGF-β_1 treatment increased the stability of R2 and R1 messages in malignant cells following exposure to TGF-β_1, indicating that TGF-β_1 is capable of regulating R2 and R1 mRNA levels in these cells through a mechanism of post-transcriptional stabilization. This observation is consistent with observations in BALB c/3T3 mouse fibroblasts, in which mRNA *cis–trans* interactions that regulate R1 and R2 message stability have recently been described [Chen et al., 1993; Amara et al., 1993, 1994]. The prevailing hypothesis is that growth factors such as TGF-β_1 exert their biological modifications by inducing an altered program of gene expression through transcriptional activation of genes. In this regard, we suggest that the

TGF-β_1-mediated post-transcriptional stabilization of R2 and R1 mRNA may represent a component of an altered growth regulatory program associated with malignant transformation.

Mammalian ribonucleotide reductase expression has previously been shown to be modulated by TPA treatment [Choy et al., 1989; Chen et al., 1993]. TPA activates protein kinase C [Nishizuka, 1986]. Furthermore, prolonged exposure of mammalian cells to TPA has been shown to result in a downregulation of PKC activity [Young et al., 1987]. TPA treatment of C3 cells causes a rapid but transient elevation of both R2 and R1 gene expression, with R2 and R1 mRNA levels returning to unstimulated levels following prolonged exposure to TPA. These results are consistent with previous observations obtained with BALB/c 3T3 cells [Choy et al., 1989]. The observation that the TGF-β_1-induced increases in R2 and R1 message levels can be reduced by TPA pretreatment (known to downregulate PKC activity), suggests that a PKC-dependent pathway may be involved in the TGF-β_1 modulation of ribonucleotide reductase expression in C3 cells. Additionally, it is possible that in C3 cells, following prolonged exposure to TPA, a downregulation of expression or inactivation of TGF-β receptors may result. The species of TGF-β released by cells exhibiting varying malignant potential has been determined. Parental 10T½ cells produced predominantly TGF-β_2 (80%), while virtually all the TGF-β produced by C3 cells was in the form of TGF-β_1 [Schwarz et al., 1990]. Notably, the TGF-β receptor number and affinities were previously evaluated by Scatchard analysis. C3 cells exhibited approximately normal receptor numbers (1.05×10^4) compared to the parental 10T½ line (1.87×10^4) and moderately increased receptor affinity (Kd = 4.4 pM in C3 cells versus Kd = 12.3 pM in parental 10T½ cells) [Schwarz et al., 1990]. It would be interesting to determine if TPA pretreatment of C3 cells results in alterations in TGF-β receptor expression, consequently contributing to the abrogation of the TGF-β induced increase in ribonucleotide reductase gene expression.

Mammalian ribonucleotide reductase has also been shown to be subject to regulation by the tumor promoters and protein phosphatase inhibitors okadaic acid and calyculin A [Hurta and Wright, 1992b]. These studies showed that ribonucleotide reductase, particularly the R2 component, plays a fundamental role in the critical early events involved in the process of tumor promotion and illustrated a role for cellular protein phosphatases in the regulation of ribonucleotide reductase [Hurta and Wright, 1992b]. Inhibition of protein phosphatases allows the unopposed activity of protein kinases constitutively present in the cell and leads to enhanced phosphorylation of many of the substrates of protein kinases [Sassa et al., 1989]. Downregulation of PKC activation abrogated the okadaic acid mediated elevations of ribonucleotide reductase mRNAs consistent with the involvement of this signal pathway in the regulation of ribonucleotide reductase and the effects of okadaic acid [Hurta and Wright, 1994]. Downregulation of protein phosphatases by okadaic acid resulted in an abrogation of the TGF-β_1-induced elevations of R2 and R1 mRNA levels, suggesting a possible role for protein phosphatases in the signaling pathway. Interestingly, in human keratinocytes, growth arrest induced by TGF-β_1 is accompanied by protein phosphatase activation [Gruppuso et al., 1991]. The specifics of the intracellular signaling pathways responsible for the TGF-β_1-directed modulation of ribonucleotide reductase gene expression and the possible role of protein phosphatases in this process remain to be further elucidated.

Several studies have demonstrated that G-proteins may participate in transducing the mitogenic signal of polypeptide growth factors [Chambard et al., 1987; Fischer and Schonbrunn, 1988; Luttrel et al., 1988]. Furthermore, multiple G-protein-dependent and -independent transducing pathways are responsible for mediating the biological signal of TGF-β_1 [Howe et al., 1989]. In keeping with this idea, possible pertussis-toxin sensitive G-proteins may play a role in the TGF-β_1-mediated alterations of ribonucleotide reductase gene expression in highly malignant H-*ras*-transformed cells. The nature of the specific G-protein(s) and the signaling mechanism(s) involved are unknown and subject to further investigation. Interestingly, in NIH-3T3 fibroblasts (minimally responsive to TGF-β_1) when stably transfected with guanine nucleotide-binding protein $A_{i\alpha 1}$ cDNA, TGF-β_1 induced a morphological transformation; these $G_{i\alpha 1}$ transfectants also exhibited mitogenic hyperresponsiveness to TGF-β_1 [Kataoka et al., 1993]. Notably, this response to TGF-β_1 is blocked by pertussis toxin. The data reported in this study are consistent with these and other observations [Kataoka et al., 1993; Howe et al., 1989].

Clearly, the present investigation of TGF-β_1 regulation of ribonucleotide reductase and DNA synthesis in normal and malignant cells indicates that malignant H-*ras*-transformed cells contain alterations in signal transduction pathways that lead to novel regulation of ribonucleotide reductase gene expression. The observation of a relationship between H-*ras* expression and the inducibility of R1 and R2 gene expression by TGF-β_1 is consistent with the idea that *ras*-mediated activities play an essential role in this process [Wright et al., 1990b] and others [Hurta and Wright, 1994]. Determination of the precise nature of the regulatory mechanisms involved and how they interact to control ribonucleotide reductase in normal cells, as well as how this regulation is altered or abrogated in malignant cells poses, a significant challenge. Studies to address these questions are under way.

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REFERENCES

Albert DA, Nodzenski E (1989): M2 subunit of ribonucleotide reductase is a target of cyclic AMP-dependent protein kinase. J Cell Physiol 138:129–136.

Amara FM, Chen FY, Wright JA (1993): A novel transforming growth factor-β_1 responsive cytoplasmic trans-acting factor binds selectively to the 3′-untranslated region of mammalian ribonucleotide reductase R2 mRNA: Role in message stability. Nucleic Acid Res 21:20, 4803–4809.

Amara FM, Chen FY, Wright JA (1994): Phorbol ester modulation of a novel cytoplasmic protein binding activity at the 3′-untranslated region of mammalian ribonucleotide reductase R2 mRNA and role in message stability. J Biol Chem 269:6709–6715.

Bjorklund S, Skog S, Tribukait B, Thelander L (1990): S-phase specific expression of mammalian ribonucleotide reductase R1 and R2 subunit mRNAs. Biochemistry 29: 5452–5458.

Brunner AM, Marquardt H, Malacko AR, Lioubin MN, Purchio AF (1989): Site-directed mutagenesis of cysteine residues in the proregion of the transforming growth factor β_1 precursor. J Biol Chem 264:13660–13664.

Chan AK, Litchfield DW, Wright JA (1993): Phosphorylation of ribonucleotide reductase R2 protein: In vivo and in vitro evidence of a role for $p34^{cdc2}$ and CDK2 protein kinases. Biochemistry 32:12835–12840.

Chambard JC, Paris S, L'Allemain G, Pouyssegur J (1987): Two growth factor signalling pathways in fibroblasts distinguished by pertussis toxin. Nature 326:800–803.

Chen FY, Amara FM, Wright JA (1993): Mammalian ribonucleotide reductase R1 mRNA stability under normal and phorbol ester stimulating conditions: Involvement of a cis–trans interaction at the 3′untranslated region. EMBO J 12:3977–3986.

Choy BK, McClarty GA, Wright JA (1989): Transient elevation of ribonucleotide reductase activity, M2 mRNA and M2 protein in BALB c/3T3 fibroblasts in the presence of 12-*O*-tetradecanoylphorbol-13-acetate. Biochem Biophys Res Commun 162:1417–1424.

Cohen P, Holmes CFB, Tsukitani Y (1990): Okadaic acids: A new probe for the study of cellular regulation. Trends Biochem Sci 15:98–102.

Cory J, Whitford L Jr (1972): Ribonucleotide reductase and DNA synthesis in Ehrlich ascites tumor cells. Cancer Res 32:1301–1306.

Egan SE, McClarty GA, Jarolim L, Wright JA, Spiro I, Hager G, Greenberg AH (1987a): Expression of H-*ras* correlates with metastatic potential: Evidence for direct regulation of the metastatic phenotype in 10T½ and NIH 3T3 cells. Mol Cell Biol 7:830–837.

Egan SE, Wright JA, Jarolim L, Yanagihara K, Bassin RH, Greenberg AH (1987b): Transformation by oncogenes encoding protein kinases induces the metastatic phenotype. Science 238:202–205.

Felgner LL, Gadek LR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielson M (1987): Lipofection: A highly efficient, lipid mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84:7413–7417.

Fischer JB, Schonbrunn A (1988): The bombesin receptor is coupled to a guanine nucleotide binding protein which is insensitive to pertussis and cholera toxins. J Biol Chem 263:2808–2816.

Gentry I, Lisubin E, Purchio AF, Marquardt H (1988): Molecular events in the processing of recombinant type 1 pre-pro-transforming growth factor beta to the mature polypeptide. Mol Cell Biol 8:4162–4168.

Gough NM (1988): Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. Anal Biochem 173:93–95.

Greenberg AH, Egan SE, Wright JA (1989): Oncogenes and metastatic progression. Invasion Metast 9:360–378.

Gruppuso PA, Mikumo R, Brautigan DL, Braun L (1991): Growth arrest induced by transforming growth factor β_1 is accompanied by protein phosphatase activation in human keratinocytes. J Biol Chem 266:3444–3448.

Hards RG, Wright JA (1981): *N*-Carbamoyloxyurea-resistant Chinese hamster ovary cells with elevated levels of ribonucleotide reductase activity. J Cell Physiol 106:309–319.

Howe PH, Bascom CC, Cunningham MR, Leof EB (1989): Regulation of transforming growth factor β_1 action by multiple transducing pathways: Evidence for both G-protein-dependent and -independent signalling. Cancer Res 49:6024–6031.

Hurta RAR, Wright JA (1990): Mammalian drug resistant mutants with multiple gene amplifications: Genes encoding the M1 component of ribonucleotide reductase, the M2 component of ribonucleotide reductase, ornithine decarboxylase, p5-8, the H-subunit of ferritin and the L-subunit of ferritin. Biochim Biophys Acta 1087:165–172.

Hurta RAR, Wright JA (1992a): Alterations in the activity and regulation of mammalian ribonucleotide reductase by chlorambucil, a DNA damaging agent. J Biol Chem 267: 7066–7071.

Hurta RAR, Wright JA (1992b): Regulation of mammalian ribonucleotide reductase by the tumor promoters and protein phosphatase inhibitors okadaic acid and calyculin A. Biochem Cell Biol 70:1081–1087.

Hurta RAR, Wright JA (1994): Alterations in the cyclic AMP signal transduction pathway regulating ribonucleotide reductase gene expression in malignant H-*ras* transformed cell lines. J Cell Physiol 158:187–197.

Hurta RAR, Samuel SK, Greenberg AH, Wright JA (1991): Early induction of ribonucleotide reductase gene expression by TGF-β_1 in malignant H-*ras* transformed cell lines. J Biol Chem 266:24897–24100.

Jakowlew SB, Kondaiah P, Flanders KC, Thompson NL, Dillard PJ, Sporn MB, Roberts AB (1988): Increased expression of growth factor mRNAs accompanies viral transformation of rodent cells. Oncogene Res 2:135–148.

Kataoka R, Sherlock J, Lanier SM (1993): Signalling events initiated by transforming growth factor β_1 that require $G_{i\alpha 1}$. J Biol Chem 268:19851–19857.

Kim S-J, Angel P, Lafyatis R, Hattori K, Kim KY, Sporn MB, Karin M, Roberts AB (1990): Auto-induction of transforming growth factor β_1 is mediated by the AP-1 complex. Mol Cell Biol 10:1492–1497.

Le A, Ferrell GA, Dishon DS, Le QQ-A, Sifers RN (1992): Soluble aggregates of the human $PiZ_{\alpha 1}$-antitrypsin variant are degraded within the endoplasmic reticulum by a mechanism sensitive to inhibitors of protein synthesis. J Biol Chem 267:1072–1080.

Lewis WH, Kuzik BA, Wright JA (1978): Assay of ribonucleotide reduction in nucleotide-permeable hamster cells. J Cell Physiol 94:287–298.

Luttrel LM, Hewlett EL, Romero G, Rogol AD (1988): Pertussis toxin-treatment attenuates some effects of insulin in BC3H-1 murine myocytes. J Biol Chem 263:6134–6141.

McClarty GA, Chan AKM, Wright JA (1986): Characterization of a mouse cell line selected for hydroxyurea resistance by a stepwise procedure: Drug dependent overproduction of ribonucleotide reductase. Somatic Cell Mol Genet 12:121–131.

McClarty GA, Chan AK, Engstrom Y, Wright JA, Thelander L (1987): Elevated expression of M1 and M2 components and drug-induced post-transcriptional modulation of ribonucleotide reductase in a hydroxyurea resistant mouse cell line. Biochemistry 26:8004–8011.

McClarty GA, Chan AK, Choy BK, Wright JA (1990): Increased ferritin gene expression is associated with increased ribonucleotide reductase gene expression and the establishment of hydroxyurea resistance in mammalian cells. J Biol Chem 265:7539–7547.

Mukherjee R, Molloy GR (1987): 5,6-Dichloro-1-β_1-D-ribofuranosylbenzimidazole inhibits transcription of the β-hemoglobin gene in vivo at initiation. J Biol Chem 262:13697–13705.

Nicolson GL (1984): Generation of phenotypic diversity and progression in metastatic tumor cells. Cancer Metast Rev 3:25–42.

Nishizuka Y (1986): Studies and perspectives of protein kinase C. Science 233:305–312.

Pelech SL, Sanghera JS, Daya-Makin M (1990): Protein kinase cascades in meiotic and mitotic cell cycle control. Biochem Cell Biol 68:1297–1330.

Penttinen RP, Kobayashi S, Bornstein P (1988): Transforming growth factor β increases mRNA for matrix proteins both in the presence and in the absence of changes in mRNA stability. Proc Natl Acad Sci USA 85:1105–1108.

Pertovaara L, Sistonen L, Bos TJ, Vogt PK, Keski-Oja J, Alitalo K (1989): Enhanced *jun* gene expression is an early genomic response to transforming growth factor β stimulation. Mol Cell Biol 9:1255–1262.

Phillips DR, Crowthers DM (1986): Kinetics and sequence specificity of drug–DNA interactions: An in vitro transcription assay. Biochemistry 25:7355–7362.

Raghow R (1987): Regulation of messenger RNA turnover in eukaryotes. Trends Biochem Sci 12:358–360.

Ralph RK, Darkin-Rattray S, Scholfield P (1990): Growth-related protein kinases. Bioessays 12:121–124.

Roberts AB, Thompson NL, Heine U, Flanders C, Sporn MB (1988): Transforming growth factor-β: Possible roles in carcinogenesis. Br J Cancer 57:594–600.

Samuel SK, Hurta RAR, Kondaiah P, Khalil N, Turley EA, Wright JA, Greenberg AH (1992): Autocrine induction of tumor protease production and invasion by a metallothionein-regulated TGF-β_1 (Ser 223, 225). EMBO J 11:1599–1605.

Sassa T, Richter WW, Uda N, Suganuma M, Suguri H, Yoshizuwa S, Hirota M, Fujiki H (1989): Apparent activation of protein kinases by okadaic acid class tumor promoters. Biochem Biophys Res Commun 159:939–944.

Schwarz LC, Gingras M-C, Goldberg G, Greenberg AH, Wright JA (1988): Loss of growth factor dependence and conversion of transforming growth factor-β_1 inhibition to stimulation in metastatic H-*ras* transformed murine fibroblasts. Cancer Res 48:6999–7003.

Schwarz LC, Wright JA, Gingras M-C, Kondaiah P, Danielpour D, Pimental M, Sporn MB, Greenberg AH (1990): Aberrant TGF-β production and regulation in metastatic malignancy. Growth Factors 3:115–127.

Sporn MB, Roberts AB (1990): TGF-β: Problems and prospects. Cell Regul 1:875–882.

Steeper JR, Stuart CD (1970): A rapid assay for CDP reductase activity in mammalian cell extracts. Anal Biochem 34:123–130.

Tamm I, Sehgal PB (1978): Halobenzimidazole ribosides and RNA synthesis of cells and viruses. In Lauffer MA, Bang FB, Maramorosch K, Smith KM (eds): "Advances in Virus Research." San Diego: Academic Press, pp 187–258.

Thelander L, Erickson S, Akerman M (1980): Ribonucleotide reductase from calf thymus. Separation of the enzyme into two nonidentical subunits, proteins M1 and M2. J Biol Chem 255:7426–7432.

Ullman B, Gudas LJ, Cleft SM, Martin DW Jr (1979): Isolation and characterization of purine nucleoside phosphorylation-deficient T-lymphoma cells and secondary mutants with altered ribonucleotide reductase: A genetic model for immunodeficiency disease. Proc Natl Acad Sci USA 76:1074–1078.

Wakefield LM, Smith DM, Masui T, Harris CC, Sporn MB (1987): Distribution and modulation of the cellular receptor for transforming growth factor β. J Cell Biol 105:965–975.

Weber G (1983): Biochemical strategy of cancer cells and design of chemotherapy: G.H.A. Clowes Memorial Lecture. Cancer Res 43:3466–3492.

Weinberg RA (ed) (1989): "Oncogenes and the Molecular Origins of Cancer." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 46–66.

Weinberg G, Ullman B, Martin DW Jr (1981): Mutator phenotypes in mammalian cell mutants with distinct biochemical defects and abnormal deoxyribonucleoside triphosphate pools. Proc Natl Acad Sci USA 78:2447–2451.

Wilder PJ, Rizzino A (1991): Effects of transforming growth factor β on the anchorage-independent growth of murine epithelial JB6 cells. Cancer Res 51:5898–5902.

Wright JA (1989): Altered mammalian ribonucleoside diphosphate reductase from mutant cell lines. Int Ency Pharmacol Ther 128:89–111.

Wright JA, Chan AK, Choy BK, Hurta RAR, McClarty GA, Tagger AY (1990a): Regulation and drug resistance mechanisms of mammalian ribonucleotide reductase and the significance to DNA synthesis. Biochem Cell Biol 68:1364–1371.

Wright JA, Egan SE, Greenberg AH (1990b): Genetic regulation of metastatic progression. Anticancer Res 10:1247–1256.

Wright JA, Turley EA, Greenberg AM (1993): Transforming growth factor beta and fibroblast growth factor as promoter of tumor progression to malignancy. Crit Rev Oncogenesis 4:473–492.

Young S, Parker PJ, Ullrich A, Stabel S (1987): Down regulation of protein kinase C is due to an increased rate of degradation. Biochem J 244:775–779.