Inhibitory Effect of 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole on a Protein Kinase*

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The adenosine analog 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibits specific in vitro transcription initiation by RNA polymerase II. We report here that DRB inhibits a protein kinase present in an extract of HeLa cells and does not inhibit other protein kinases contained in the same extract. The protein kinase affected by DRB is cyclic AMP independent, prefers acidic protein substrates such as casein and phosphitin, and utilizes GTP as the phosphate donor almost as effectively as ATP in the phosphorylation reaction. The DRB-sensitive protein kinase is also stimulated by polyamines and inhibited by quercitin and heparin. The biochemical and chromatographic properties of this enzyme correspond to those used in experiments in vivo. In HeLa cells, DRB is able to inhibit in vivo phosphorylation on some nuclear proteins. In HeLa cell extracts, in vitro phosphorylation of several proteins by [γ-32P]GTP is inhibited by DRB. This protein kinase has a DRB sensitivity profile identical to the one previously reported for specific in vitro transcription by RNA polymerase II in a whole-cell extract (Zandomeni, R., Mittleman, B., Bunick, D., Ackerman, S., and Weinmann, R. (1982) Proc. Natl. Acad. Sci. U. S. A. 79, 3167-3170). Thus we suggest that this protein kinase mediates DRB inhibition of specific RNA polymerase II transcription in vivo and in vitro.

The nucleotide analog DRB1 specifically inhibits the synthesis of eukaryotic heterogeneous nuclear RNA in insect and mammalian cells; and almost no cytoplasmic messenger RNA is found in DRB-treated cells (for a review, see Ref. 1). In eukaryotes, RNA polymerase II is responsible for RNA transcription of heterogeneous nuclear RNA, the precursor of mature mRNA. Purified RNA polymerase II cannot accurately initiate transcription on different DNA or chromatin templates containing the necessary promoter recognition sequences; the resulting RNAs are initiated nonspecifically at nicks or at ends (2). Accurate initiation is achieved when proteins from crude cell extracts in addition to DNA promoter sequences and RNA polymerase II are present (3-5). Thus, factors distinct from pure RNA polymerase II are also required for specific in vitro initiation of transcription and can be separated from crude cell extracts (6-10) by chromatography into several fractions distinct from the RNA polymerase II. None of these essential transcriptional factors has yet been structurally or functionally identified.

Purified RNA polymerase II-nonspecific transcription is not affected by DRB at the concentrations that inhibit RNA synthesis in vivo (11). In contrast, we have reported that DRB inhibits in vitro transcription in systems that retain the fidelity of initiation. The inhibitory concentrations are identical to those in experiments in vivo. Inhibition of specific transcription in this mutant (12). Our demonstration of DRB sensitivity of the whole-cell extract transcripts in contrast to the lack of effect on purified RNA polymerase II and our results with the DRB8 cell mutant support the notion that the action of DRB is mediated by a transcriptional factor.

We reported a requirement for hydrolysis of the βγ bond of ATP for faithful initiation in vitro of whole-cell extracts (13, 14). The ATP analog adenyl-5′-yl imidodiphosphate which has a nonhydrolyzable βγ-phosphate bond cannot substitute for ATP during initiation, although it is utilized during elongation of specific transcription (13, 14). DRB also acts as an inhibitor of initiation in vitro since preinitiated RNA molecules are able to elongate after treatment with DRB (14, 15). The similarity of action of DRB and adenyl-5′-yl imidodiphosphate on in vitro initiation of specific transcription prompted us to test whether DRB could act via a protein kinase, a reaction known to require hydrolysis of the βγ bond of ATP. In the present studies, we have identified and characterized a kinase activity affected by DRB.

**Experimental Procedures**

**Materials**

Nucleotides and Radiochemicals—ATP, GTP, and cAMP were obtained from P-L Biochemicals; [γ-32P]ATP, [γ-32P]GTP, and [32P]orthophosphate were obtained from Amersham Corp.

Protein Substrates—Hydrolyzed and partially dephosphorylated casein, phosphitin, calf thymus histone, and BSA were purchased from Sigma.

Column Chromatography—DEAE-Sepharose was from Pharmacia. Phosphocellulose P-11 was obtained from Whatman. Resins were washed following the manufacturer’s instructions. All other reagents were of the highest grade obtainable.

**Methods**

Protein Kinase Assay—Routine kinase determinations were performed at 30 °C during 30 min in 20 mM HEPES, pH 7.9, 60 mM

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1 The abbreviations used are: DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; BSA, bovine serum albumin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.
**DRB Inhibits a Casein Kinase Type II**

KCl, 7 mM MgCl₂, 0.1 mM dithiothreitol, 10 μM [γ-32P]ATP or [γ-32P]GTP (1000 dpm/pmol) with 2 mg/ml casein as substrate acceptor in a total volume of 20 μl. Reactions were stopped by spotting half of the reaction mixture on Whatman 3MM paper and submerging in a total volume of 20 μl. Filters were extensively washed for 15 min each in four or five changes of 5% trichloroacetic acid, once in 95% ethanol, and air dried. Filters were counted in a Beckman LS liquid scintillation counter. Values were corrected for phosphorylation on endogenous proteins when this activity exceeded 2% of the activity in the presence of casein.

**Protein Determination**—Protein concentration was estimated by the Lowry's Blue dye-binding method of Bradford (16), with BSA as standard.

**Cells and Preparation of Extract**—Whole-cell extracts from HeLa cells were prepared according to Manley et al. (4). The whole-cell extracts had protein concentrations of 15 mg/ml. Extracts were frozen and stored in liquid nitrogen.

**DEAE-Sepharose Chromatography**—The whole-cell extract was thawed, and the KC concentration was adjusted to 50 mM and applied to a DEAE-Sepharose column (2 ml/ml of extract) previously equilibrated in buffer A (200 mM HEPES, 0.2 mM EDTA, 1 mM dithiothreitol, and 20% glycerol) containing 50 mM KCl. After washing with the same buffer (2 bed volumes) the activity was eluted with a linear gradient (10 bed volumes) of KCl from 50 to 1000 mM. Active fractions were pooled and could be stored on ice for at least 2 months without significant loss of activity.

**Phosphocellulose Chromatography**—The pooled active fractions were concentrated by vacuum dialysis and loaded onto a phosphocellulose column (1 ml of resin/1 ml of active fractions) equilibrated in buffer A (200 mM KCl). The column was washed with 2 column volumes of starting buffer and eluted with a linear gradient (10 bed volumes) of KCl from 200 to 1000 mM. Activity eluted as a sharp peak at 620 mM KCl. Active fractions were pooled and concentrated as before and stored on ice for at least 2 months without loss of activity.

**Casein Labeling**—Casein (0.2 mg) was incubated with 10 μCi of [γ-32P]GTP and protein kinase obtained from the phosphocellulose column. Unincorporated ribotriphosphates were separated by Sephadex G-75 chromatography, and the kinase was heat inactivated (50°C).

**Thin-layer Chromatography**—Nucleotides were separated by polyethylenimine thin-layer chromatography developed in 1 M LiCl. Chromatograms were exposed at -70°C with Fuji RX film and Lightning Plus intensifying screens.

**[32P]Orthophosphate Labeling of HeLa Cells**—HeLa cells grown to log phase were concentrated to 10 × 10⁶ cells/ml and preincubated for 15 min with or without 60 μM DRB. The cells were washed once with Joklik's phosphate-free medium, 25 mM HEPES, pH 7.5. The cells were resuspended in the same medium (1 ml containing 500 μCi/ml of [32P]orthophosphate (carrier free, Amersham Corp.) and incubated at 37°C with or without 60 μM DRB for the times indicated. Following incubation the cells were rinsed in two changes of phosphate-buffered saline.

**Nuclei Isolation**—The preparation of nuclei from labeled HeLa cells was carried out as described by Wray et al. (17). Nuclei were further separated through two sucrose cushions.

**Extraction of Proteins and Electrophoresis**—Total nuclear proteins were extracted as described by LeStoungue and Beyer (18). Electrophoresis in 10% SDS-polyacrylamide gels was as described by Laemmli (19). Gels were stained with Coomassie Brilliant Blue R-250 and visualized with a UV illuminator (19). Chromatograms were exposed at -70°C with Fuji RX film and Lightning Plus intensifying screens.

**In Vitro Phosphorylation of Endogenous Protein Contained in the Whole-cell Extract**—The conditions for phosphorylation of proteins contained in the whole-cell extract were the same as for in vitro transcription (4). Incubations at different concentrations of DRB (0, 5, and 45 μM) were for 10 min at 30°C with [γ-32P]GTP as the labeling nucleotide. Reactions were terminated by the addition of 4 volumes of ethanol. Pellets were resuspended in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 5% 2-mercaptoethanol), heated for 5 min at 95°C, and analyzed on 10% SDS-polyacrylamide gels (19).

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**RESULTS**

**DRB Affects a Phosphotransferase Activity Contained in the HeLa Whole-cell Extract**

To analyze kinase activities, in vitro phosphorylation experiments were performed using the whole-cell extract as a source of activity and partially hydrolyzed and dephosphorylated casein as the substrate acceptor. No significant inhibition of the phosphotransfer reaction (Table I) was detected when [γ-32P]ATP was used as a phosphate donor at different DRB concentrations. Using [γ-32P]GTP as the phosphate donor and casein as the acceptor, we found that half of the kinase activity of the extract could be inhibited by DRB at concentrations similar to those that affect in vitro specific transcription; at 60 μM DRB, phosphorylation on casein was reduced by 49.6% in a 30-min reaction at 30°C.

The presence of cAMP at concentrations between 10⁻⁷ and 10⁻⁴ M had no effect on the phosphotransfer reaction using [γ-32P]GTP as a donor in the presence or absence of DRB, suggesting that the DRB-sensitive protein kinase was cAMP-independent (results not shown).

**Chromatographic Separation of the HeLa Protein Kinases**

**DEAE-Sepharose Chromatography**—Whole-cell extracts active in the specific in vitro transcription system were applied to DEAE-Sepharose, and proteins were eluted with a salt gradient from 50 to 500 mM KCl (Fig. 1A). Using casein as a phosphate acceptor and [γ-32P]ATP as a donor, kinase activity was detected in the flowthrough and two major peaks (Fig. 1B). The kinase activity present in the flowthrough was occasionally absent in individual experiments, but when present, showed no sensitivity to DRB. The first peak eluted at approximately 150 mM KCl and was resistant to DRB. The second peak of kinase activity eluted at 240 mM KCl and was inhibited at an average of 50% in the presence of 45 μM DRB. When [γ-32P]GTP was used as the phosphate donor, only the second peak was detected (Fig. 1C) since the protein kinases resistant to DRB (flowthrough and first peak) and detected with [γ-32P]ATP were unable to utilize GTP in the phosphorylation reactions. The protein kinase activity that eluted at 240 mM KCl utilized ATP or GTP as the substrate donor, and the activity was sensitive to DRB with both donors (Fig. 1).

**TABLE 1**

| DRB | [γ-32P]ATP | [γ-32P]GTP |
|-----|------------|------------|
|     | pmol/30 min | % of control | pmol/30 min | % of control |
| μM  |             |            |             |            |
| 0   | 20.15       | 100        | 9.3         | 100        |
| 1   | 21.2        | 105.2      | 9.1         | 97.5       |
| 3   | 19.9        | 98.7       | 8.7         | 93.3       |
| 10  | 19.5        | 96.8       | 8.6         | 92.2       |
| 30  | 13.1        | 94.8       | 7.1         | 76.1       |
| 60  | 18.9        | 93.8       | 4.8         | 51.4       |

**Effect of DRB on Protein Kinase activity in the whole-cell extract**

Protein kinase activity was measured under transcriptional conditions using the whole-cell extract as enzyme source, [γ-32P]ATP (3000 cpm/pmol) or [γ-32P]GTP (3000 cpm/pmol) as phosphate donor, and casein as a phosphate acceptor. Different concentrations of DRB were present during the incubations as indicated. Values were corrected for phosphorylation on endogenous proteins, representing approximately 10% of the activity in the presence of casein when ATP was used as donor. Values were not corrected when GTP served as donor, since phosphorylation of endogenous protein in this case represented less than 2%.
Fig. 1. Chromatography of protein kinase on DEAE-Sepharose. Two ml of whole-cell extract were dialyzed against 20 mM HEPES, pH 7.9, 50 mM KCl, 1 mM dithiothreitol, 0.2 mM EDTA, and 20% glycerol. 30.6 mg of protein were applied on a DEAE-Sepharose column equilibrated in the same buffer. After washing with 2 column volumes of the same buffer, a linear gradient of 50-500 mM KCl was applied. Fractions of 1 ml were collected at a flow rate of 1 ml/min. Aliquots (4 µl) were tested for kinase activity in the absence (☐—☐) or presence of DRB (Δ—Δ). A, the protein concentrations in the fractions; B, kinase activity when [γ-32P]ATP is used as a phosphate donor; C, the kinase activity using [γ-32P]GTP as a phosphate donor.

Table II

Partial purification of DRB-sensitive protein kinase from whole-cell extract

| Protein          | Volume ml | Concentration mg/ml | Total mg | Specific activity total units* units/mg |
|------------------|-----------|---------------------|----------|----------------------------------------|
| Whole-cell extract | 2         | 15.8                | 30.6     | 8,000 261                              |
| DEAE-Sepharose   | 0.4       | 7.0                 | 2.8      | 12,500 4,464                           |
| Phosphocellulose | 0.5       | 1.2                 | 0.6      | 6,000 10,000                           |

*One unit equals 1 pmol of phosphate transferred from [γ-32P]GTP to casein in 30 min at 30°C.

1, B and C. After this first purification step, it was possible to use [γ-32P]ATP as a phosphate donor and clearly detect the DRB inhibition of the phosphorylation reaction. The enzyme sensitive to DRB represents 20-30% of the total kinase activity in the crude cell extract; the remaining 70-80% of the activity is unaffected by DRB. It is probable that the masking effect of the many other kinases that can use ATP, as compared with the one(s) that can use GTP, obscured the effect of DRB on casein phosphorylation when [γ-32P]ATP was used as a phosphate donor and the whole-cell extract as a source of phosphorylation activity. DEAE-Sepharose chromatography proved to be essential to separate or eliminate nucleotides, protein phosphatases, or endogenous inhibitors of the kinase reaction present in the whole-cell extract.

Total kinase activity increased 2- to 5-fold on average after this chromatographic step, as did the DRB-sensitive protein kinase activity which was purified 10- to 15-fold (Table II).

Phosphocellulose Chromatography—The fractions containing the DRB-sensitive kinase activity were pooled, concentrated, and subjected to ion-exchange chromatography on phosphocellulose (P-11). This step separated a small amount of DRB-resistant protein kinase remaining (Fig. 2). The DRB-sensitive protein kinase eluted at approximately 600 mM KCl. The specific activity was increased 2.5-fold upon purification by phosphocellulose chromatography, and the recovery was 48% (Table II). SDS-polyacrylamide gel electrophoresis analysis and silver staining of the proteins revealed the presence of many bands, precluding the identification of the polypeptides responsible for the kinase activity (data not shown) at this stage of purification. The chromatographic properties of the DRB-sensitive protein kinase on DEAE-Sepharose and phosphocellulose are similar to those of the previously reported casein kinase type II (21, 23, 28, 81).

Properties of the Partially Purified DRB-sensitive Protein Kinase

Sensitivity to DRB—To determine the DRB sensitivity of the protein kinase activity and compare it with that of the transcription reaction, a DRB dose-response experiment was performed. DRB-sensitive protein kinase from the phosphocellulose chromatography step was tested against different concentrations of the drug (Fig. 3). The DRB concentrations giving 50% inhibition are between 4 and 8 µM, in good agreement with the in vitro and in vivo values reported previously on transcription inhibition (11). A small fraction of residual DRB-resistant phosphorylation always remained. The CAMP-independent protein kinase that eluted in front of the DRB-sensitive protein kinase from the DEAE-Sepharose column was used as a control in the same experiment to determine the specificity of DRB inhibition (Fig. 3). The small reduction in activity of this kinase in the presence of DRB probably reflects contamination with the DRB-sensitive kinase at this early stage of purification. To rule out the possibility that DRB inhibition of phosphate incorporation
into casein is due to a stimulation of a protein phosphatase present in the protein kinase fraction. In order to test this hypothesis, we incubated 32P-labeled casein with the partially purified protein 32PCasein. As shown in Table III, no activity was detected in the presence of DRB. The whole cell extract hydrolyzed 30% of the labeled phosphate, whereas the Escherichia coli alkaline phosphatase removed 70% of the label. Similarly, to exclude a possible role for DRB-stimulated nucleotidases in the DRB inhibition of 32P incorporation into casein, we incubated [a-32P]ATP or [a-32P]GTP with the partially purified kinase and separated the soluble nucleotides by polyethyleneimine chromatography. No ATPase or GTPase activity was detected in the kinase active fractions, even in the presence of DRB. The whole cell extract hydrolyzed 30% of the labeled phosphate, whereas the Escherichia coli alkaline phosphatase removed 70% of the label. Similarly, to exclude a possible role for DRB-stimulated nucleotidases in the DRB inhibition of 32P incorporation into casein, we incubated [a-32P]ATP or [a-32P]GTP with the partially purified kinase and separated the soluble nucleotides by polyethyleneimine chromatography. No ATPase or GTPase activity was detected in the kinase active fractions, even in the presence of DRB. The whole cell extract, as a control, contains both activities (Fig. 4). Thus, the inhibition of 32P incorporation in casein by DRB is not due to either phosphatase or nucleotidase stimulation but is instead a direct effect of the drug on the protein kinase. In addition to the HeLa protein kinases shown, other protein kinases (25) were tested for their response to DRB. The cAMP-dependent protein kinase from rabbit muscle and from bovine heart and the catalytic and regulatory subunits of the cAMP-dependent protein kinase from bovine heart were incubated in the presence of casein and ATP as acceptor and donor, respectively.

**Table III**

Absence of protein phosphatase activity in the HeLa partially purified protein kinase preparation

| Protein Phosphatase Activity | cpm | % of Input |
|-----------------------------|-----|-----------|
| DRB-sensitive protein kinase | 23,100 | 97.3 |
| DRB-sensitive protein kinase plus DRB (60 μM) | 22,100 | 100.9 |
| Whole-cell extract | 15,400 | 70.3 |
| E. coli alkaline phosphatase | 6,300 | 28.9 |
| None (input) | 21,900 | 100.0 |

No changes in activity in the presence or absence of DRB were detected (results not shown). These observations indicate that the DRB-sensitive protein kinase is specifically inhibited by DRB. To further characterize this partially purified protein kinase, we analyzed the protein acceptors and some inhibitors which also affect casein kinase II.

**Protein Acceptors**—The utilization of various proteins as acceptors by the DRB-sensitive protein kinase was studied (Table IV). At saturating concentrations (1.5 mg/ml), casein and phosvitin were good acceptors relative to histone and serum albumin. DRB affected the phosphorylation of casein and phosvitin but not of histone and BSA.

**Effect of Other Agents**—The partially purified protein kinase was further characterized by testing a variety of compounds on the enzyme activity in vitro (Fig. 5). CAMP does not alter the enzyme activity at all, consistent with our results.

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**Table IV**

Phosphorylation on various protein acceptors by the DRB-sensitive protein kinase

| Protein Acceptor | Activity (pmol/30 min) |
|------------------|-----------------------|
| Casein           | Control: 12.5 ± 1.0  |
| Phosvitin        | DRB (60 μM): 4.3 (34) |
| Histone          | 1.1                   |
| BSA              | 0.3                   |

* Data are given as picomoles of 32P incorporated at 30°C during 30 min. Percentage of control is given in parentheses.
Protein Targets for Phosphorylation

**DRB Effects on the in Vitro Phosphorylation of Proteins Contained in the Whole-cell Extract**—To determine the natural targets for this phosphotransferase activity, we exploited the DRB sensitivity to analyze the acceptors both in vivo and in the whole-cell extract. Phosphorylation of the endogenous proteins contained in the whole cell extract by endogenous kinases was selectively measured with [γ-32P]GTP as the phosphate donor substrate and in the presence of different concentrations of DRB. Proteins contained in the transcriptionally active extract that are phosphate acceptors under these conditions were separated by SDS-polyacrylamide gel electrophoresis and analyzed by autoradiography (Fig. 6). A clear inhibition by increasing concentrations of DRB can be seen for the major phosphorylated proteins detected, suggesting that the protein kinase(s) present in the cell extract that utilizes GTP is(are) sensitive to DRB. Conversion of [γ-32P]GTP to [γ-32P]ATP was not observed during the 10-min incubation period. Seven phosphoproteins of 40, 50, 72, 85, 92, 95, and 200 kDa are the major endogenous protein accep-

![Fig. 5. Effect of various compounds on the DRB-sensitive protein kinase activity. The agents indicated were added at increasing concentrations to the standard reaction mixture and protein kinase activity assayed as described under “Experimental Procedures.” Results are the means of duplicate determination expressed as per cent of control.](image)

using the whole cell extract, indicating this protein kinase is indeed cAMP independent (Fig. 5A). The glycosaminoglycan heparin, a potent inhibitor of casein kinase II (20), also inhibited the DRB-sensitive protein kinase (Fig. 5B). Naturally occurring polycations, such as polyamines, have been shown to stimulate the activity of casein kinase II (21). We observed a 1.5- to 2-fold stimulation of the enzyme by spermine and spermidine (Fig. 5, C and D). The bioflavonoids quercetin and rutin have been recently described as a specific inhibitor and an inactive analog, respectively, for protein kinase type II activities (21). Accordingly, quercetin inhibits and rutin does not affect phosphorylation of casein by the DRB-sensitive protein kinase. All of these properties are similar to those previously observed with casein kinases type II (20–22).

![Fig. 6. Effect of DRB on in vitro phosphorylation of the proteins contained in the whole-cell extract. Whole-cell extracts were incubated under transcription conditions (4) in the presence of 0, 5, and 45 μM DRB. The reaction mixtures were incubated for 10 min at 30 °C using [γ-32P]GTP as a phosphate donor substrate. After incubation, the proteins were ethanol precipitated. Precipitates were collected by centrifugation, dissolved in denaturing buffer, and loaded on a 10% SDS-polyacrylamide gel run according to Laemmli (19). The figure shows an autoradiogram which displays the in vitro 32P-labeled proteins. Molecular weight markers are: phosphorylase b (92.5 kDa), BSA (68 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa).](image)
of many other protein kinases that can utilize ATP as a donor. "PIATP is used, a far more complex pattern emerges (data tors contained in the whole-cell extracts. Phosphorylation of these proteins is inhibited to the same extent under exactly the same conditions that affect in vitro transcription and at similar DRB concentrations. In a parallel reaction using [α-32P]GTP, DRB inhibition on the expected runoff RNA was observed (data not shown). The use of the crude cell extracts where cellular compartmentalization is lost and in which so many different cellular proteins are brought in contact with the protein kinase precludes any identification of molecular targets involved in in vitro transcription. Due to the presence of many other protein kinases that can utilize ATP as a donor on a wide range of proteins as substrate acceptors when [γ-32P]ATP is used, a far more complex pattern emerges (data not shown).

DRB Effects on Phosphorylation of Nuclear Proteins in Vivo—To investigate the differential effects of DRB on phosphorylation of nuclear proteins, HeLa S-3 cells were preincubated with or without 60 μM DRB for 15 min. After the preincubation period, the medium was changed to Joklik's phosphate-free medium (25 mM HEPES, pH 7.2) and pulse labeled for 3 min (Fig. 7, lanes 1 and 2) and 15 min (Fig. 7, lanes 3 and 4) with [32P]orthophosphate with (Fig. 7, lanes 2 and 4) or without (Fig. 7, lanes 1 and 3) 60 μM DRB. Nuclei were prepared, and the proteins present were analyzed by SDS-polyacrylamide slab gel electrophoresis. The autoradiogram resulting from this analysis is presented in Fig. 7. A large number of nuclear proteins was found to be phosphorylated, but few were inhibited by DRB. Only four protein species (indicated with arrows) of 37, 41, 52, and 160 kDa among those nuclear phosphoproteins were inhibited by DRB, whereas many other proteins, e.g. 19, 23.5, 32, 45, 62, and 68-kDa species, were unaffected by the drug. The effect of DRB was detectable only during short periods of incubation (3 min): 15-min or longer incubations increased the total 32P incorporation, but the effect of DRB was less evident. To establish the identity between the in vitro and in vivo labeled polypeptides requires a detailed study of the polypeptides themselves and of the phosphorylation sites within them. The important conclusion from these experiments is that DRB affects phosphorylation of some nuclear proteins under the same conditions it affects transcription, both in vitro and in vivo.

**DISCUSSION**

In this study we show that a protein kinase present in a whole cell extract is inhibited by DRB concentrations similar to those affecting specific in vitro initiation of transcription in the same extracts. This HeLa cell protein kinase, which can be partially purified based on its sensitivity to DRB, appears similar to other cyclic nucleotide-independent casein kinases described in ascites tumor cells (26), liver nuclei (27), calf thymus (28), bovine brain (29, 30), bovine adrenocortical gland (31), bovine lung (32), human erythrocytes (33), rat hepatoma (21), and Drosophila (34). All of these kinases require a similar high-salt concentration for optimal activity and tend to aggregate at low ionic strength. They also show a preference for acidic protein substrates like casein or phosphorylated RNA, function with GTP as well as with ATP as the phosphate donor with comparable apparent Km, and require the same Mg++ concentration for activity. Spermine and spermidine stimulate these kinase activities, and heparin and quercetin are potent inhibitors. The elution profile of this kinase from DEAE-Sepharose and phosphocellulose is similar to the chromatographic properties reported for casein kinase II. Furthermore, chromatography of the calf thymus enzyme, purified to homogeneity using DRB sensitivity as the only assay, showed similar profiles in hydroxyapatite and casein-agarose chromatography, as well as in subunit structure, as the previously reported calf thymus casein kinase II (28). All of these properties allow to identify this DRB-sensitive protein kinase as a casein kinase II-type enzyme.

The ability of this kinase to utilize GTP as well as ATP further identifies it as a G-type protein kinase. The function of these casein G-type protein kinases is presently unknown. The high degree of homology in these kinases among diverse phyla suggests an essential and conserved function. The elements of the transcriptional machinery are also highly conserved, since RNA polymerase II from different sources can complement the S-100 extracts in the runoff synthesis of adenovirus major late promoter RNA (3). Furthermore, DNA sequences common to eukaryotic RNA polymerase II promoters have been identified, and accurate and specific transcription of heterologous genes can be achieved using human cell extracts.

The results presented here strongly suggest a link between the DRB-induced inhibition of RNA polymerase II transcrip-
tion and that of casein kinase II. First, the DRB inhibition curves of in vivo and in vitro RNA polymerase II transcription (11) are completely superimposable on the curves of DRB inhibition of the phosphotransferase reaction catalyzed by the kinase described here. Second, the action of DRB is restricted to casein kinase II, with no inhibitory effects for other protein kinases. Moreover, no effects of DRB other than on transcription have been reported (1), with the exception of a recent study in which DRB was shown to inhibit protein phosphorylation (35).

The reports about direct stimulation of purified RNA polymerase II activity after in vitro phosphorylation have been conflicting. Stimulation of transcription by RNA polymerase II purified from a rat tumor was elicited by a nuclear protein kinase (17). However, others report no effect on purified RNA polymerase II from calf thymus when phosphorylated by calf thymus nuclear protein kinase I or II (28). These differences might be related to the level of residual phosphorylation of the polynucleotides after purification. The DRB-sensitive casein kinase we describe can phosphorylate RNA polymerase II purified from calf thymus. The polypeptide phosphorylated corresponds to the 21-kDa subunit of RNA polymerase II (29). In vitro phosphorylation would depend not only on the subcellular localization of both enzymes. Whereas the notion of a kinase activity that preferentially modulates the level of transcription initiation via phosphorylation/dephosphorylation of RNA polymerase II itself is attractive, it is equally possible that the phosphorylation reaction occurs on other transcriptional factors involved in one of the several steps of transcription initiation. Further studies are required to discriminate between these possibilities and to determine whether the DRB effect on transcription is mediated through its inhibitory properties on the kinase.

The results of the present study strengthen our previous reports that DRB affects the activity of one of the transcriptional factors involved in accurate and specific transcription (12). The DRB-sensitive kinase activity can use GTP as the phosphate donor and thus appears to be unrelated to the ATP requirement (13). In fact, GTP was present in the experiments that demonstrated the ATP requirement (13) and was available for phosphorylation by the endogenous casein kinase II contained in whole-cell extracts. Thus, phosphorylation by the DRB-sensitive kinase and hydrolysis of the \( \beta\gamma \) bond of ATP by an ATPase or another kinase may be independent requirements for faithful transcription initiation by RNA polymerase II in vitro.

Experiments to establish a link between the activity of this kinase and natural targets that could function either as transcriptional factors or the RNA polymerase II itself were hampered by technical difficulties. First, the systems used for in vitro transcription contain a multiplicity of kinases, nucleotidases, etc. Second, because these are extracts in which the subcellular structures are destroyed, the proximity of the natural protein substrates for the kinases is altered, and thus other proteins or other sites on the same proteins might be phosphorylated. Third, in vivo experiments we cannot adequately control: the precursor into which the radiolabel incorporates; the particular kinase(s) involved; or the phosphorylation of a particular protein by different kinases at the same or different sites. Despite these difficulties, we have detected inhibition of phosphorylation by DRB of some proteins both in the whole-cell extracts and in vivo. The phosphate levels on all the major phosphorylated proteins detected in the whole-cell extracts were reduced in the presence of DRB. Thus the endogenous protein kinase(s) activity(ies) able to use GTP was inhibited by DRB. We have shown here that the major kinase activity able to use GTP was inhibited by DRB and corresponds to casein kinase II in chromatographic and biochemical properties. In vivo inhibition of phosphorylation of nuclear proteins was detected only after very short labeling periods. Although transcription is inhibited for longer times, the nature of the phosphorylation assay used (see above) does not allow us to detect an effect after 15 min. Similar results on inhibition of phosphorylation of nuclear proteins by DRB were independently obtained by Egyhazi et al. (35) while this report was in preparation. Not only were they able to detect DRB-sensitive phosphorylation of a 40- to 41-kDa polypeptide but they were also able to immunoprecipitate this protein using antibodies directed against the stimulatory factor SI1 (35, 36). Monospecific antibodies directed against SI1 inhibit specific RNA polymerase II-mediated transcription (36). We postulate that the conversion of SI1 to its phosphorylated SI1' form (37) is thus mediated by the DRB-sensitive protein kinase II or G-casein kinase described here, based on our finding that casein kinase II is inhibited by DRB at concentrations similar to those required for inhibition of transcription initiation. Studies are in progress to firmly establish the relationship between DRB inhibition of specific transcription, the role of SI1 and SI1', and the inhibition by DRB of the casein kinase described here.

In conclusion, the evidence presented here supports the notion that the DRB-sensitive kinase acts as an essential factor for RNA polymerase II transcription.

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