Phosphorylation of Some Chromosomal Nonhistone Proteins in Active Genes is Blocked by the Transcription Inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)

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ABSTRACT The distribution of rapidly phosphorylated chromosomal proteins between chromosome I, chromosome II + III, chromosome IV, and nuclear sap including the matrix was investigated in salivary gland cells of *Chironomus tentans*. Chromosome IV, which carries most active nonribosomal genes in the cell, was found to be enriched in four rapidly phosphorylated nonhistone polypeptides (M_r = 25,000, 30,000, 33,000, and 42,000) in parallel with the transcriptional activity rather than with the DNA content of the chromosome. Also the histones H2A and H4 are rapidly phosphorylated but the phosphorylation is proportional to the DNA content of each chromosome sample. The ^{32}P-labeled M_r = 42,000 polypeptide immunologically cross-reacted with an antibody elicited against the transcription stimulatory factor S-II isolated from Ehrlich ascites tumor cells (Sekimizu, K., D. Mizuno, and S. Natori, 1979, *Exp. Cell Res.*, 124:63–72). In addition, indirect immunofluorescence studies on chromosome IV with antisera against the stimulatory factor II revealed a selective staining of the active gene loci. The incorporation of ^{32}P into three chromosome IV nonhistone polypeptides, especially into the M_r = 42,000 polypeptide, was lowered by 70–85% shortly after administration of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a likely inhibitor of heterogeneous nuclear RNA transcription at initiation level. The possibility of a causal relationship between inhibited phosphorylation of chromosomal proteins and blocked transcription of heterogeneous nuclear RNA genes by DRB is discussed.

The nature of events involved in regulation of eukaryotic gene expression at the transcriptional level is still to a large extent unknown. The recognition of initiation sites and the sequence of events involved in the step of transcription initiation play, in all likelihood, a crucial role in the exertion of gene control (for review, see reference 8). An important regulatory potential lies in the structure of chromatin. Unfolding of tightly packed chromatin fibers at selected gene loci may facilitate the finding of initiation sites by RNA polymerase molecules and transcription factors, and may guide the initiation reaction itself. Transcriptional regulatory potential may also lie in the concentration and chemical modification of RNA polymerase and in specific transcription factors that may act independently on, or in cooperation with, RNA polymerase. Thus the identification of molecules and molecular assemblies with regulatory function and the elucidation of the significance of postsynthetic protein modifications as phosphorylation, acetylation, methylation, etc. in gene control require analysis of structural as well as catalytic constituents of the chromatin (for reviews, see references 42 and 46). On the basis of the involvement of posttranslational protein phosphorylation in many cellular regulatory mechanisms (for reviews, see references 20 and 37) and because of the observed modulation of specific chromosomal phosphoproteins in transcription studies, a coupling between the phosphorylation/dephosphorylation of specific nuclear proteins and transcriptional activity has been proposed (22, 29, 31).

We recently characterized a dozen rapidly phosphorylated

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nuclear proteins (RPNP)[1] of which two were tentatively identified as histones H2A and H4 (17). Individual RPNP were found to be bound to chromatin more or less tightly but the fractionation procedure used did not permit an appropriate separation of chromosomal and extrachromosomal RPNP; chromosomal distribution of RPNP in relation to DNA content and gene activity could not be investigated. In the present study we used the isolation technique described by Sass (35) to collect unfixed individual polytene chromosomes, in particular chromosome IV, which harbors the giant tissue specific Balbiani rings (BRs) (2, 6, 34). Transcriptional modulation by selective inactivation of heterogeneous nuclear RNA (hnRNA) genes was induced by the nucleoside analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (for review, see reference 44). The inhibitory effect of DRB is noticeable within minutes (12) and it leads to a reformation of thick chromosome fiber in parallel with the repression of hnRNA genes (1). An interference of DRB with an initiation event was concluded from studies of transcription in intact *Chironomus* cells (13, 16), in isolated DNA and extract of HeLa cells in vitro (49, 50), and in isolated nuclei of mammalian origin (48). An alternate mechanism of action, implying an enhanced premature termination of transcripts, was reported by other investigators (19, 26, 43).

We report here results showing that chromosome IV with only ~11% of the total nuclear DNA content (5) contains 26–29% of the total chromosomal fraction of four ^{32}P-labeled nonhistone polypeptides (M_r = 25,000, 30,000, 33,000, and 42,000) in agreement with the transcriptional activity of chromosome IV (7). The phosphorylation of three of these polypeptides was largely lowered in the course of inactivation of hnRNA genes by DRB, whereas the incorporation of ^{32}P into histones H2A and H4 was elevated under identical experimental conditions.

MATERIALS AND METHODS

Labeling Conditions: Salivary glands were isolated from fourth instar larvae of the dipteran *Chironomus tentans* (2). The glands were dissected from 50–100 6–8-wk-old animals and explanted into phosphate-free Cannon medium (33) ("HEPES-Cannon", a modified Cannon medium without phosphate and with 14 mM HEPES + NaOH to pH 7.2) and incubated for a total of 25 min at 20°C. The glands were then transferred into 100–200 μl of HEPES-Cannon medium containing 20 mCi/ml of [^{32}P]orthophosphate (carrier-free, The Radiochemical Centre, Amersham, England) and incubated for 10 min at 20°C. In inhibition experiments, salivary glands were preincubated with 65 μM DRB or 20 μg α-amanitin/ml. A stock solution of DRB at 5 mg/ml 70% ethanol was used. Before incubation, an appropriate volume of the stock solution was evaporated to dryness and DRB was subsequently solubilized in HEPES-Cannon. After incubation, the incorporation was stopped by transferring the glands into nuclear isolation medium at 0°C.

Isolation and Fractionation of Nuclei: Chromosome I, chromosome II + III, and chromosome IV were prepared after isolation and purification of nuclei from labeled salivary gland cells as described by Sass (35) with minor modifications. Nuclei isolated in Sass medium (8 mM NaCl, 90 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 15 mM Sörensen phosphate buffer, pH 6.0, and 0.2% Nonidet P-40 (NP-40). In addition, 1 mM NaF and 1 mM phenylmethylsulfonylfluoride (PMSF) were included to inhibit phosphatase and proteolytic activities. The nuclei were washed free of cytoplasmic debris and transferred into a clean dish with fresh medium. They were then broken open in a new volume of isolation medium by the pipetting of small groups of nuclei (2–5 nuclei per pipetting) with a narrow capillary pipette. The diameter of the tip was approximately two-thirds of the nuclear diameter. All glasswares were coated with Sigmacote (Sigma Chemical Co., St. Louis, MO) and dried at 50°C overnight. The free chromosomes were collected in separate groups containing chromosome I, chromosome II + III, and chromosome IV. The nuclear material remaining after removal of chromosomes was also collected and analyzed.

Extraction of Proteins: Unfractionated nuclei or subsets of chromosomes were sonicated in 100 μl of solution with low salt content (1.5 mM KCl, 1 mM NaF, and 1 mM PMSF) for 45 s at 0°C as described (17). After sonication, 25 μl of SDS sample buffer (100 mM Tris·PO_4, pH 7.0, 20 mM EDTA, and 3% SDS) and 5 μl of glycerol were added to the homogenate. The mixture was then concentrated in vacuo over H_2SO_4 to a volume of ~25 μl, which takes 40 min at 20°C. Finally, 5 μl of mercaptoethanol was added and the extract was heated for 2 min at 100°C before electrophoresis.

Electrophoresis: One-dimensional electrophoresis was run in a 12% SDS polyacrylamide slab gel as described by Laemmli and Favre (25). After the electrophoretic run, gels were fixed in 50% methanol + 10% acetic acid for 30 min and stained with silver (21). The autoradiogram of gels were made on Kodak X-Omat AR film with KYOKKO Super HS intensifying screens at 4°C.

Immunoprecipitation: Polyclonal antisera directed against RNA polymerase II from *Chironomus thummi* (24) were a gift from Dr. E. K. F. Bautz (University of Heidelberg) and the antisera raised against the stimulatory factor S-II purified from Ehrlich ascites tumor cells (38) were a gift from Dr. S. Natori (University of Tokyo).

Nuclei were isolated from ^{32}P-labeled cells and subsequently sonicated in lysis buffer (17). After centrifugation at 30,000 *g* for 5 min, the buffer in the supernatant was changed to 10 mM Tris·HCl buffer, pH 7.2, containing 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, and 0.5% NP-40 by filtration through a Bio-Gel P-6DG (Bio-Rad Laboratories, Richmond, CA) column. Various portions of the ^{32}P-labeled protein extract were then mixed with 10–25 μl of antisera to S-II or to RNA polymerase II and the mixtures were incubated for 60 min at 20°C. ^{32}P-labeled extracts incubated with and without preimmune sera were used as controls. Antigen-antibody complexes were recovered by binding to 50 μl of protein A-Sepharose CL-4B beads (Pharmacia Fine Chemicals Div., Uppsala, Sweden, 50% slurry) for 30 min at 20°C. The complexes were washed three times in 150 μl of the same buffer containing 100 μg ovalbumin/ml. The antigen-antibody complexes were dissociated in a solution of 100 mM Tris·PO_4 buffer, pH 7.2, 20 mM EDTA, 5% mercaptoethanol, and 3% SDS by being heated at 100°C for 5 min, the sample was cooled, and the supernatant was subjected to electrophoresis in a 12% SDS polyacrylamide gel.

Immunofluorescence Microscopy: Chromosome IV was prepared from isolated nuclei and stained by an indirect method essentially as described by Sass (35, 36). Chromosome IV pipetted onto a nonsiliconed slide were washed and subsequently fixed with 1.75% formaldehyde for ~4 min. Next, the slides were transferred to PBS (140 mM NaCl, 15 mM Sörensen phosphate buffer, pH 7.0) at 4°C and the chromosomes were washed for ~30 min. They were then reacted with 10 μl of antisera or with preimmune sera for 30 min at 20°C with a dilution of 1:5. After extensive washing in PBS, the reaction was repeated with 10 μl of fluorescein isothiocyanate-labeled swine anti-rabbit IgG (Dako Corp., Santa Barbara, CA) with a dilution of 1:30. Following thorough washing in PBS, the chromosomes were mounted in glycerol:PBS, 1:9, under a coverslip and the fluorescence of the chromosomes was examined and photographed with a Zeiss photomicroscope equipped with a dark-field oil condensor and a KP 500 excitation filter. Photographs were taken with Tri-X film which was developed in HC-110 developer (Kodak).

RESULTS

Chromosomal Distribution of RPNP

To obtain uniform labeling of all RPNP, we incubated salivary glands for 10 min with $^{32}P_i$, in accordance with previous findings (17). Nuclei were isolated from labeled cells and subsets of chromosomes I, chromosomes II + III, chromosomes IV, and of the nonchromosomal nuclear material were collected and subsequently extracted to release the proteins (Fig. 1). The autoradiograph from the electrophoretic separations in a 12% SDS polyacrylamide gel of material extracted from chromosome I (lane *A*), chromosome IV (lane *C*), and from the nonchromosomal fraction (lane *D*) displays ^{32}P-labeled bands corresponding to nonhistone polypeptides with the apparent M_r values of 22,000, 25,000, 30,000, 33,000, 42,000, 44,000, 65,000, and 95,000 (17). The radioactivity patterns usually exhibit the M_r = 22,000 and 44,000 polypep-

[1] *Abbreviations used in this paper:* BR(s), Balbiani ring(s); DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole; hnRNA, heterogeneous nuclear RNA; NP-40, Nonidet P-40; PMSF, phenylmethylsulfonyl fluoride; RPNP, rapidly phosphorylated nuclear proteins.

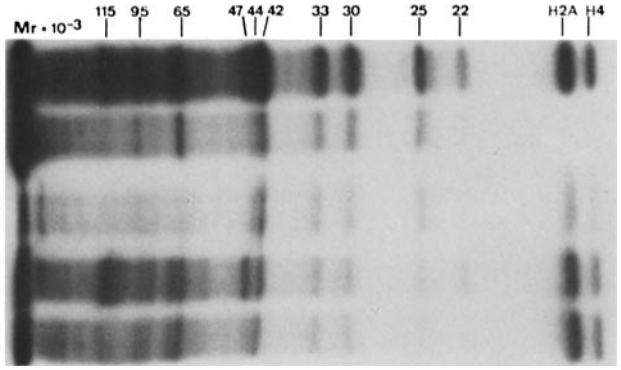

FIGURE 1 Electrophoretic separation of ^{32}P-labeled RPNP derived from chromosome I (lane *A*), chromosome II + III (lane *B*), chromosome IV (lane *C*), nonchromosomal nuclear material (lane *D*), and from unfractionated nuclei (lane *E*). Salivary glands were preincubated in HEPES-Cannon for 25 min followed by incubation for 10 min in identical medium containing $^{32}P_i$. The glands were then transferred into isolation medium at 0°C and the nuclei were isolated. The isolated nuclei free of cytoplasmic contaminations were broken open in a new volume of fresh medium by pipetting with a narrow capillary pipette, and free chromosomes and the nonchromosomal nuclear material were collected in separate groups: 80 chromosomes I, 55 chromosomes II + III, 105 chromosomes IV, and 95 nonchromosomal material were isolated. For comparison a group of 60 unfractionated nuclei was isolated. The samples were sonicated for 45 s at 0°C in 100 μl of a low salt solution containing 1.5 mM KCl, 1 mM PMSF, and 1 mM NaF. After sonication, the proteins were solubilized in SDS sample buffer and the extract was heated for 2 min at 100°C before electrophoresis. Electrophoresis was carried out in a 12% SDS polyacrylamide gel. After the electrophoretic run, the gel was dried and autoradiographed on Kodak X-Omat AR film at 4°C. For other data, see Materials and Methods.

tides with variable intensities. The electrophoretic profile of chromosome II + III proteins (lane *B*) shows the presence of two prominent ^{32}P-labeled M_r = 47,000 and 115,000 polypeptides which are not detectable in the other nuclear fractions. Since the extract of chromosome II + III contains material from nucleolar organizer genes with associated nulceoli, it appears likely that the M_r = 47,000 and 115,000 polypeptides are exclusively of nucleolar origin. In addition to the nonhistone phosphoproteins, the labeling patterns of chromosome I (lane *A*), chromosome II + III (lane *B*), and chromosome IV (lane *C*) exhibit the presence of histones H2A and H4 which are absent in the nonchromosomal nuclear material (lane *D*). The identification of H2A and H4 has been confirmed by electrophoresis in acetic acid-urea-Triton gels (unpublished data). The electrophoretic separation of proteins from unfractionated nuclei (lane *E*) is included for comparison.

The distribution of ^{32}P label in the nonhistone M_r = 25,000, 30,000, 33,000, and 42,000 polypeptides and in histones H2A and H4 was quantitated from the electrophoretic separations by densitometer scanning of autoradiograms. Table I shows the intranuclear distribution of ^{32}P-labeled M_r = 25,000, 30,000, 33,000, and 42,000 polypeptides and histones H2A and H4 between chromosome I, chromosome II + III, chromosome IV, and the nonchromosomal nuclear material. The interchromosomal distribution of DNA, determined by Daneholt and Edström (5), is shown for comparison. The results listed in Table I reveal that the relative distributions of the M_r = 25,000, 30,000, 33,000, and 42,000 polypeptides between the analyzed nuclear fractions bear a close resemblance. Of the total nuclear fraction of the M_r = 25,000, 30,000, 33,000, or 42,000 polypeptides, 19–24% is bound to chromosome I, 23–28% to chromosome II + III, 17–20% to chromosome IV, and 30–35% is found in the nonchromosomal nuclear material. The relative distribution of ^{32}P-labeled histones H2A and H4 differs markedly from that of the four nonhistone polypeptides (34–36% is associated with chromosome I, 54–55% with chromosome II + III, and 10–11% with chromosome IV). Thus the interchromosomal distribution of ^{32}P-labeled core-histones closely follows that of DNA (Table I).

The specific activities (expressed as ^{32}P-labeled protein/μg of DNA) of M_r = 25,000, 30,000, and 33,000 polypeptides agree reasonably well within each chromosome but differ between different chromosomes (Table II). The specific activities per unit weight of chromosomal DNA for the three polypeptides were calculated to be 22–27, 15–18, and 58–65 on chromosome I, chromosome II + III, and chromosome IV, respectively. The corresponding figures for the M_r = 42,000 polypeptide are two- to threefold higher in each chromosome fraction. Furthermore, the representation of the M_r = 42,000 polypeptide in the extract derived from chromosome IV is almost five times higher than in that obtained from chromosome II + III (Table II). The specific activities for histones H2A and H4, unlike the M_r = 25,000, 30,000, and 33,000 polypeptides, are of the same order on all chromosomes and are thus distributed essentially in proportion to the DNA content. However, histone H2A incorporates two to three times more ^{32}P than histone H4 per unit weight of DNA in all chromosomes (Table II).

Attempts were made to isolate and separately analyze RPNP associated with BR 1 and BR 2 on chromosome IV. Since the isolation of individual BRs from unfixed chromosome IV is beset with considerable difficulties, we dissected out BRs by means of micromanipulation (11) from isolated and partially fixed chromosome IV (70% ethanol) after the

TABLE I

Intranuclear Distribution of ^{32}P-labeled Polypeptides

Chromosomes	Polypeptides						
	25,000	30,000	33,000	42,000	H2A	H4	DNA*
I	24	21	24	19	36	34	31
II + III	23	27	28	28	54	55	58
IV	20	17	18	19	10	11	11
Nonchromosomal material	33	35	30	34	0	0	0

The experimental conditions were as described in the legend to Fig. 1. The data were obtained from densitometer scans of autoradiograms of gel profiles. The distribution of polypeptides are expressed in percent of the sum of label derived from chromosomes I, chromosome II + III, chromosome IV, and from the nonchromosomal nuclear material. The values represent the mean values of three independent experiments.

* Values taken from Daneholt and Edström.

TABLE II

Comparison between DNA Content and Amount of ^{32}P-labeled Phosphoproteins Derived from Chromosome I, Chromosome II + III, and Chromosome V

Chromosomes	^{32}P (arbitrary units)/μg DNA					
	25,000	30,000	33,000	42,000	H2A	H4
I	22	24	27	63	137	46
II + III	15	15	18	38	111	36
IV	65	59	58	180	113	45

The calculations are based on the material presented in Table I.

cells were labeled for 10 min with $^{32}P_i$. This procedure did not permit the dissection of pure BR material but gave rise to a chromosome IV fraction that was largely enriched in BR 1 and BR 2. The electrophoretic separation of BR 1 plus BR 2 proteins and the subsequent estimation of ^{32}P content of the M_r = 42,000 polypeptide and of histone H2A revealed that the ratio between the nonhistone and histone protein components was 6.5, which is nearly four times higher and 20 times higher than those obtained from analysis of unfractionated chromosome IV and chromosome II + III proteins, respectively.

The Effect of the Transcription Inhibitors DRB and α-Amanitin on Postsynthetic ^{32}P-Incorporation

In earlier works we presented evidence that DRB (14, 16) and α-amanitin (12, 15) selectively block the RNA polymerase II–promoted transcription in explanted salivary gland cells in *Chironomus* by interference with the initiation and elongation step, respectively. Using similar experimental design, hnRNA genes were suppressed by DRB and the phosphorylation of histone and nonhistone proteins were examined. Initially, the effect of DRB on ^{32}P-incorporation was analyzed in protein extracts derived from unfractionated nuclear material. Salivary glands were preincubated in 65 μM DRB for a short period (2–3 min), to minimize the possibility of secondary effects on phosphorylation, and subsequently labeled with $^{32}P_i$ for 10 min in the continued presence of DRB (Fig. 2). The nuclear proteins were extracted in an urea and NP-40–containing buffer (17), which is able to solubilize a major part of the ^{32}P-labeled nonhistone proteins, but only a limited portion of the core histones is released. The autoradiogram from the separation of the ^{32}P-labeled proteins in 12% SDS polyacrylamide gel shows a markedly lowered ^{32}P-incorporation (20–30% of control values) into the M_r = 30,000, 33,000, and 42,000 polypeptides in DRB-treated cells (Fig. 2). The labeling of the M_r = 25,000 polypeptide as well as of other nonhistone RPNP was not significantly influenced during the inhibition experiments.

The question of whether the event of transcription inhibition of hnRNA-producing genes is in itself capable of inducing alterations in phosphorylation of nuclear phosphoproteins was studied by the replacement of DRB with α-amanitin in an otherwise similar inhibition experiment. Since penetration of α-amanitin across the plasma membrane is a relatively slow process, glands were preincubated with α-amanitin for 25 min followed by labeling with $^{32}P_i$ for 10 min in the continued presence of inhibitor. As seen in Fig. 3, the electrophoretic analyses of nuclear proteins extracted in urea and NP-40–containing buffer in 12% SDS polyacrylamide gel could not reveal any significant difference between ^{32}P-labeled RPNP derived from normal and α-amanitin treated cells, in agreement with previous similar studies (17). Thus the interference with the expression of hnRNA genes at the elongation level does not appear per se to be an effector of postsynthetic ^{32}P-incorporation.

The possibility that DRB might be an effector of the stability and/or export to the cytoplasma of the M_r = 30,000, 33,000, and 42,000 nuclear phosphoproteins rather than an effector of their phosphorylation was also investigated. Salivary glands were incubated in DRB for 15 min and the nuclear proteins (including histones) were extracted and separated by electrophoresis. The distribution of silver-stained nuclear proteins were then compared with that of nuclear proteins derived from untreated cells. The M_r = 30,000, 33,000, and 42,000 phosphoproteins could be identified as small but distinct bands in the staining profiles of both control and inhibited cells. As seen in Fig. 4, the patterns closely coincide, indicating that DRB exerts no major effect on the turnover and/or transport to the cytoplasm of the M_r = 30,000, 33,000, and 42,000 phosphoproteins.

In view of the enrichment of chromosome IV in the M_r = 30,000, 33,000, and 42,000 polypeptides and of the results obtained in Figs. 2 and 4, the effect of DRB on phosphorylation of chromosome IV proteins was investigated. Subsets of chromosome IV were collected from DRB-treated and untreated cells and the patterns of phosphorylation were compared. Salivary glands were preincubated with DRB for 10 min and labeled in the presence of $^{32}P_i$ and DRB for another 10 min; nuclei were isolated and chromosome IV were col-

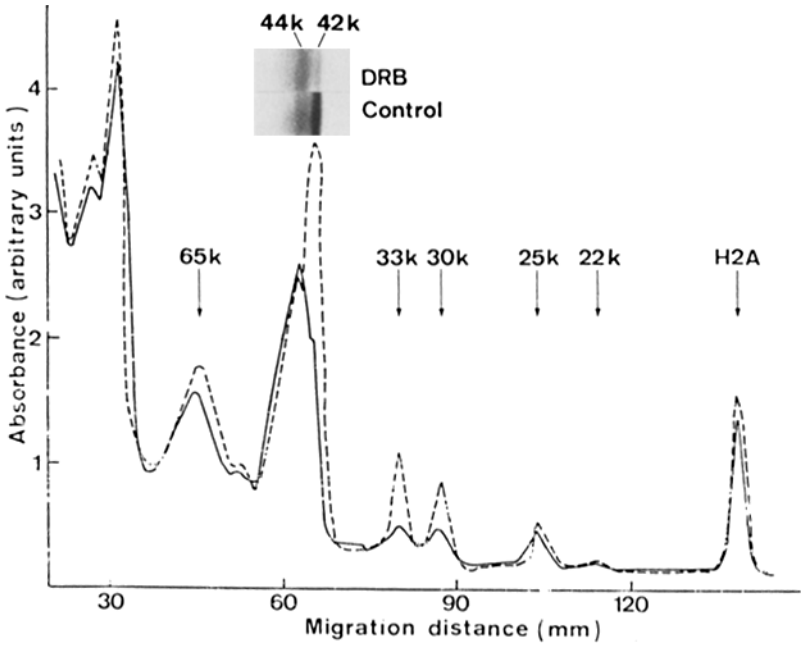

FIGURE 2 Electrophoretic analyses of RPNP after labeling in the presence and absence of DRB. 50 glands were preincubated for 2–3 min in medium containing 65 μM DRB. They were then transferred to another volume of the same medium supplemented with the inhibitor and $^{32}P_i$, and were incubated for 10 min. Control glands were labeled in the absence of DRB in an otherwise parallel procedure. After incubation, nuclei were isolated, sonicated, the homogenate was centrifuged at 18,000 *g* for 10 min, and the pellet was extracted with lysis buffer. To the lysis buffer extract SDS was added (3% final concentration) and the sample was kept for 60 min at 20°C. The buffer was changed to a fourfold diluted SDS buffer by filtration through a Bio-Gel P2 column. Electrophoresis was carried out in a 12% SDS polyacrylamide gel. For more details, see the legend to Fig. 1 and Materials and Methods. The scans of electrophoretic profiles of control (- - -) and DRB-treated (——) cells. *Inset:* the M_r = 40,000–45,000 range of the autoradiogram representing label from control (*bottom*) and DRB- (*top*) treated cells. *k*, × 10^3.

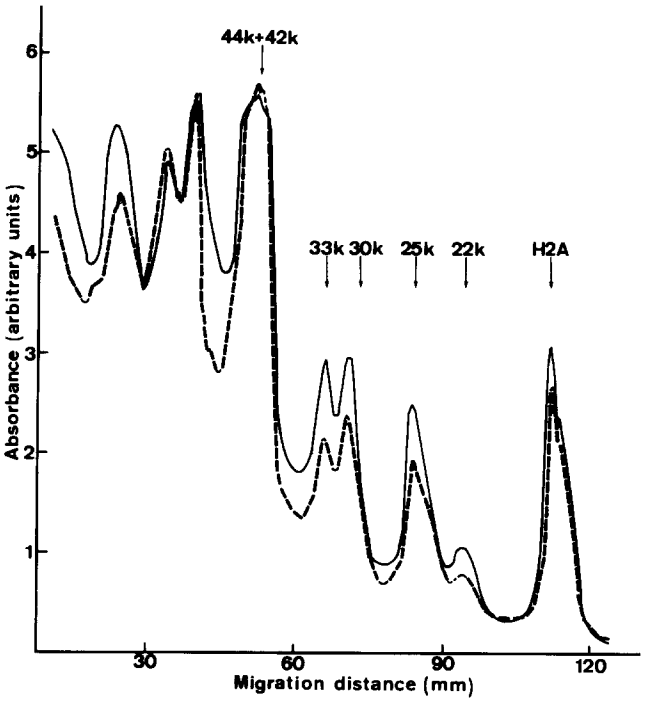

FIGURE 3 Electrophoretic analyses of RPNP after labeling in the presence and absence of α-amanitin. 50 glands were preincubated for 25 min in media containing α-amanitin 20 μg/ml. They were then transferred to another volume of the same medium with α-amanitin and $^{32}P_i$, and were incubated for 10 min. Control glands were labeled in the absence of DRB in an otherwise parallel procedure. For other data, see the legend to Fig. 2 and Material and Methods. Densitometer scans of control (- - -) and α-amanitin-treated (——) cells. *k*, × 10^3.

lected (Fig. 5). Control glands were labeled in the absence of DRB in an otherwise parallel procedure. The chromosomal protein extracts were separated in a 12% SDS polyacrylamide gel. The densitometer tracings of the autoradiogram of ^{32}P-labeled polypeptides exhibited a considerable reduction in incorporation of ^{32}P (15–30% of control value) into the M_r = 30,000 and 33,000 polypeptides and an almost complete abolishment of labeling in the M_r = 42,000 polypeptide (Fig. 5). The altered phosphorylation of these polypeptides was in marked contrast to the phosphorylation patterns of histones H2A and H4 which showed a 2.5- to threefold increase in phosphorylation under the same conditions in accordance with our previous observations (18). While the addition of nucleoside analogue DRB to explanted salivary gland cells induced an immediate modulation of rapidly turned over phosphate groups, associated with the M_r = 30,000, 33,000, and 42,000 polypeptides, which coincided in time with the onset of the transcription inhibition, the effect on histone phosphorylation was significant only after 3–10 min of preincubation with DRB (Fig. 2 and reference 18).

The M_r = 42,000 Phosphoprotein Shows Immunological Relatedness to a Polypeptide That Stimulates hnRNA Transcription In Vitro

Three immunologically related and RNA polymerase II–associated transcription factors (M_r = 24,000, 40,500, and 41,000) have been isolated and extensively characterized (30). The M_r = 41,000 factor (S-II′) appeared to be the phosphorylated form of an M_r = 40,500 polypeptide (S-II) (39). The significance of the covalently bound phosphate group(s) in S-II is, however, unclear at the present time. Antibody raised against S-II blocks accurate α-amanitin-sensitive DNA transcription (30). Immunofluorescence studies showed that nuclear protein(s) in both salivary gland cells of flesh-fly larvae and in HeLa cells cross-react with S-II suggesting an evolutionary conservation of S-II (38). This observation together with a close resemblance in molecular size between S-II (M_r = 40,500) and our M_r = 42,000 polypeptide suggested the possibility of a structural and functional relationship between the two. We have addressed this question by studying the immunoreactivity between antibody elicited against S-II from Ehrlich ascites tumor cells (38) and our ^{32}P-labeled phosphoproteins from *Chironomus*. Fig. 6 shows the autoradiograph of ^{32}P-labeled polypeptides immunoprecipitated by anti–S-II sera and preimmune sera from electrophoresis in a 12% SDS polyacrylamide gel. As seen in Fig. 6, only one ^{32}P band (lane *C*), corresponding to the M_r = 42,000 phosphoprotein, reacted with the antibody although there is a weak, disperse background labeling in the higher molecular weight range of the pattern. The preimmune sera did not react significantly with any ^{32}P-labeled material (lane *B*). The electrophoretic profile of ^{32}P-labeled proteins that were not subjected to immunoreaction is shown for comparison (lane *A*). Occasionally, the electrophoretic pattern of immunoprecipitated material exhibited the presence of additional very weak bands representing the M_r = 25,000, 30,000, and 33,000 phosphopeptides. In conclusion, the immunological experiments with antiserum raised against S-II point to the existence of an immunological relatedness between the structure of the M_r = 42,000 phosphoprotein in *Chironomus* and that of the transcription stimulatory factor S-II isolated from Ehrlich ascites tumor cells.

Immunofluorescence studies have been carried out on isolated chromosome IV to detect the chromosomal localization and abundance of antigenic determinants that eventually cross-react with the S-II antisera. Fig. 7*C* shows that the indirect fluorescence appearing after incubation with anti-S-II sera is on the whole restricted to the transcriptionally active BR regions. The condensed band region on chromosome IV remained virtually unstained. The size of the puffs appears to be correlated with the intensity of staining. Incubation of isolated chromosome IV with preimmune sera resulted in a weak indirect immunofluorescence although the BRs are slightly more stained than the non-BR regions (Fig. 7*B*).

The phosphorylation of RNA polymerase II subunit(s) has been found to be correlated with an increased rate of gene expression, and the RNA polymerase II activity is stimulated by nuclear cyclic-nucleotide independent protein kinase II (23). Phosphate-acceptor smaller subunit(s) of polymerase II were identified in yeast cells (24,000 and 33,500 subunits) (3) and in calf thymus and HeLa cells (20,500 subunit) (4). Thus, phosphorylated RNA polymerase II subunits in size ranges similar to those of our M_r = 25,000 and 33,000 phosphoproteins seem to occur in eukaryotic organisms implying that these polypeptides might constitute integrated subunits of RNA polymerase II in *Chironomus*. This possibility was investigated by means of immunoprecipitation with antisera directed against RNA polymerase II from *Chironomus thummi* (24). The electrophoretic analyses of the immunoprecipitated ^{32}P-labeled RPNP did not show any appreciable quantities of radioactive material from antigen-antibody complexes, which suggests the lack of common antigenic determinants between RNA polymerase II and ^{32}P-labeled RPNP

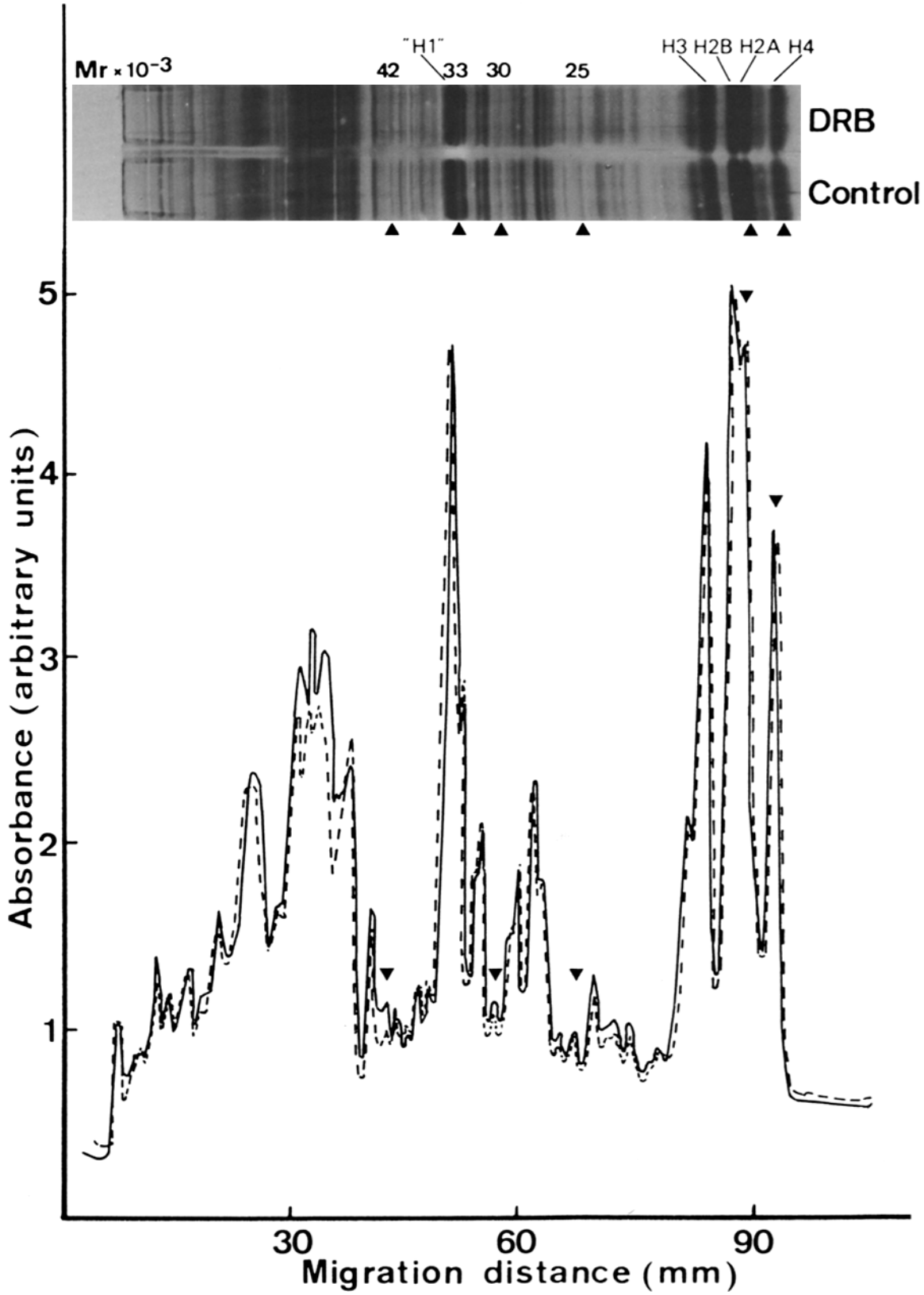

FIGURE 4 Electrophoretic analyses of nuclear proteins after incubation in the presence and absence of DRB. 20 salivary glands were incubated for 15 min in medium containing 65 μM DRB in parallel with another group of 20 glands that were kept in DRB-free medium. Nuclei were extracted with SDS sample buffer, the extracts were subjected to electrophoresis, and the protein bands were visualized by silver staining. For other data, see Materials and Methods. Densitometer scans of control (- - -) and DRB-treated (——) cells. *Inset:* autoradiograms of control (*bottom*) and DRB-treated cells (*top*). Migration is from left to right. The position of identified ^{32}P-labeled proteins is indicated by arrowheads.

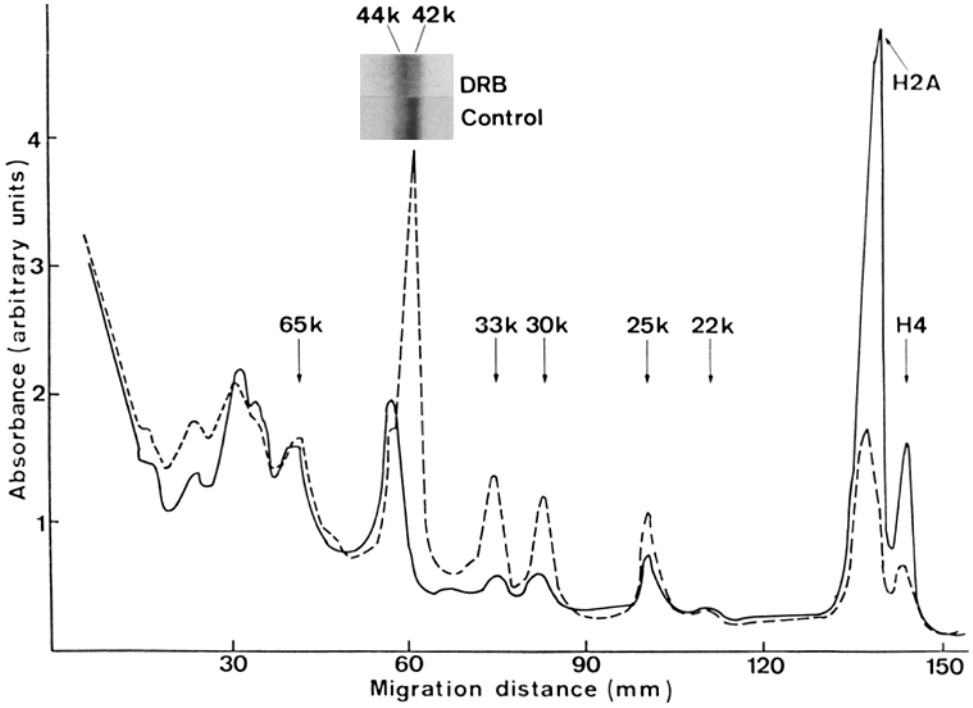

FIGURE 5 Electrophoretic analyses of chromosome IV phosphoproteins after labeling in the presence and absence of DRB. Salivary glands were incubated for 10 min in medium containing 65 μM DRB. They were then transferred to another volume of the medium supplemented with DRB and $^{32}P_i$, and incubated for 10 min. Control glands were labeled in the absence of DRB in an otherwise parallel procedure. 150 chromosomes IV per sample were sonicated, extracted, and electrophoresed in a 12% SDS polyacrylamide gel as described in Materials and Methods. The scans of control (- - -) and DRB-treated (——) cells. *Inset:* the M_r = 40,000–45,000 range of the autoradiogram representing labeled proteins from control (*bottom*) and DRB-treated (*top*) cells. *k*, × 10^3.

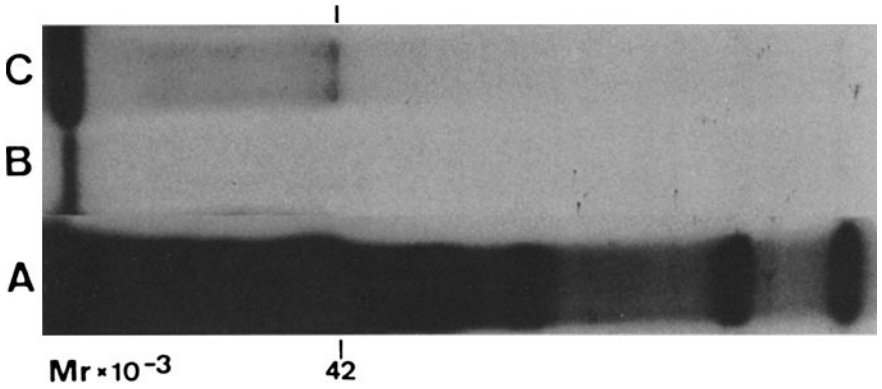

FIGURE 6 Electrophoretic analyses of immunoprecipitated ^{32}P-labeled RPNP after reaction with antisera raised against the stimulatory factor S-II and with preimmune sera. ^{32}P-labeled protein extract derived from isolated nuclei was divided into three equal portions. To one sample 10 μl of antisera and to another 10 μl of preimmune sera were added and the mixtures were incubated. The third portion of the ^{32}P-labeled extract was electrophoresed without further treatment. The antigen-antibody complexes were bound to protein A-Sepharose CL-4B and the complexed antigens were dissociated by being heated in an SDS-containing solution. The electrophoresis was carried out in a 12% SDS polyacrylamide gel. For more details, see Materials and Methods. Untreated control, (lane *A*), preimmune sera (lane *B*), antisera (lane C).

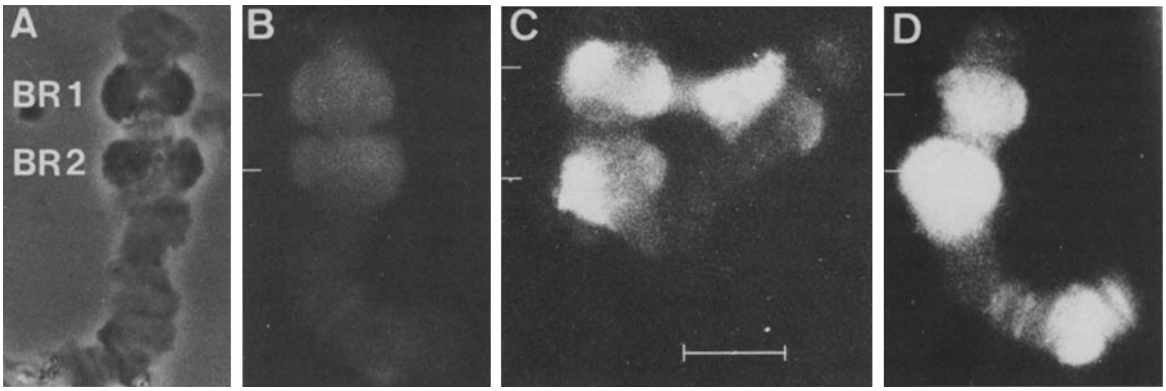

FIGURE 7 Fluorescence distribution on isolated chromosome IV after immunological cross-reaction with antisera elicited against stimulatory factor S-II (*C*) and RNA polymerase II (*D*) and with fluorescein isothiocyanate-labeled swine anti-rabbit IgG. Phase-contrast (*A*) and fluorescence (*B*) micrographs after treatment with preimmune sera are controls. Bar, 25 μm. × 540.

(data not shown). Nevertheless, the immunofluorescence experiment with anti-RNA polymerase II and isolated chromosome IV produced a preferential staining of the BR regions of chromosome IV and of a few minor puffs (Fig. 7*D*) in accordance with the results of Sass (36). The fluorescence distribution pattern obtained with anti-polymerase II sera mimicked that obtained with anti-S-II sera; both were positively correlated with gene activity on chromosome IV.

DISCUSSION

The result presented in this communication provide new information on a group of four rapidly phosphorylated nonhistone proteins (M_r = 25,000, 30,000, 33,000, and 42,000) that possess a number of interesting properties in common. We reported previously that their phosphorylation is posttranslational, independent of ongoing protein synthesis and attaining steady-state labeling with $^{32}P_i$ within 10 min in intact cells (17). The present analyses revealed that all of the four phosphorylated nonhistone polypeptides have similar relative distributions between chromosome I, chromosome II + III, chromosome IV, and the nonchromosomal nuclear material. Another point of consensus is their intrachromosomal partition which appears to be proportional to the transcriptional activity rather than to the DNA content of the chromosomes. From earlier reports it can be inferred that chromosome IV with only ~11% of the nuclear DNA content manufactures ~25% of hnRNA (5, 6). In turn, BR 1 + BR 2 on chromosome IV represent ~0.5% of chromosome IV DNA (47) but produce >90% of chromosome IV RNA (13). Thus, it is evident from these data that the transcriptional activity is correlated with puff size rather than with DNA content. The enrichment in ^{32}P-labeled M_r = 25,000, 30,000, 33,000, and 42,000 polypeptides on chromosome IV, comprising 29, 26, 26, and 29%, respectively, of the total chromosomal fractions, is also relatively well correlated with the transcriptional activity of chromosome IV. The specific significance of the nonhistone M_r = 25,000, 30,000, 33,000, and 42,000 phosphoproteins for transcriptional activity of the chromosomes is further indicated by the finding that the amount of incorporated label into histones H2A and H4 obtained from various chromosomes is not correlated with gene activity and puff size. The distribution of these ^{32}P-labeled polypeptides is approximately proportional to the DNA and puff content of the chromosomes.

The identification of specific nonhistone chromosomal proteins that are involved in rendering relevant genetic loci transcribable by RNA polymerase II is of crucial importance for the understanding of gene regulation. As yet, it is difficult to ascribe a specific chromosomal function to the present category of closely related phosphoproteins. The preferential association of the M_r = 25,000, 30,000, 33,000, and 42,000 polypeptides with transcriptionally engaged gene loci, and their rapid dephosphorylation in the course of suppression of transcription by DRB, is, however, consistent with an involvement of these phosphopeptides in activation and/or maintaining of activity of hnRNA genes.

Most of newly synthesized and growing hnRNA transcripts have been found to be complexed to a discrete set of nonhistone proteins which are thereby intimately coupled to the transcription process and active chromatin (for review, see reference 27). These hnRNP proteins are assumed to be the site for hnRNA processing. The present category of RPNP in the M_r = 25,000–42,000 range from *Chironomus* showing a preferential accumulation in active gene loci (Balbiani rings) partially overlaps with the M_r range of core hnRNP proteins described in various cell types and organisms (10, 27). However, a direct comparison between the two is difficult to make owing to the observed variation in the hnRNP protein components reported by different investigators (10, 27, 45). Of the core hnRNP proteins in mouse erythroleukemia cells, the M_r = 32,500 and 41,500 polypeptides mimic in size our 33,000 and 42,000 phosphoproteins, respectively (10). Furthermore, the two largest components of the sextet of core hnRNP proteins (M_r = 42,000 and 44,000) in another system were found to be phosphoproteins (27). It therefore appears possible that the *Chironomus* M_r = 42,000 phosphoprotein or a portion of it is a potential core hnRNP protein. However, a direct analysis of the phosphorylated protein components of hnRNP particles in *Chironomus* cells will be required, to finally establish an eventual relationship between the M_r = 25,000–42,000 phosphoproteins and the hnRNP protein subunits.

On the basis of our immunological studies, we do not favor the possibility that ^{32}P-labeled RPNP constitute integrated subunit(s) of RNA polymerase II but leave open the question of a possible association of RPNP with functioning polymerase molecules. It is well documented that several factors present in cell extracts are necessary for accurate in vitro initiation by RNA polymerase II (28). It has also been demonstrated that a binding of transcription factor(s) to the promotor regions may take place in the absence of RNA polymerase II (9). A number of prominent features of the M_r = 42,000 phosphoprotein, like cross-reactivity with the stimulatory factor S-II, its preferential accumulation in transcriptionally active puffs, and rapid response to DRB, make it seem a likely candidate for having a function in expression of hnRNA genes. Even if the mechanism of action of S-II, including its phosphorylation to S-II′, is still obscure, the antibody raised against S-II inhibits accurate initiation of transcription from adenovirus-2 major late promotor in a HeLa cell lysate (30), and the same antibody cross-reacts with the M_r = 42,000 phosphopeptide from *Chironomus* cells.

Available information about the significance of postsynthetic phosphorylation of specific nuclear proteins lends credence to the idea that gene regulation may be brought about by phosphorylation/dephosphorylation of preexisting chromosomal nonhistone proteins (22, 42). The experimental results suggest that stimulation of phosphorylation leads to enhanced transcriptional activity and dephosphorylation to gene repression (29, 31). In the present study we found both inhibition and stimulation of protein phosphorylation in response to DRB regimen. While the phosphorylation of some chromosomal proteins of nonhistone type was impaired, the phosphorylation of histones H2A and H4 was elevated. Modifications in incorporation of ^{32}P into protein molecules in general may be induced by changes in purine nucleoside triphosphate pools, [γ-^{32}P]ATP or [γ-^{32}P]GTP, from which the postsynthetic phosphorylation of proteins is fed with label and by affecting the function of protein kinases. Considering that DRB exerted inhibitory as well as stimulatory effects on phosphorylation in the course of the same DRB regimen, it seems improbable that pool alteration could be the underlying cause of modulated ^{32}P-incorporation. The observed alteration in phosphorylation pattern would require compartmentation of ATP or GTP pools at the chromosomal or even the nucleosomal level. An alternate model assuming that DRB acts as an effector of protein kinase activity can better be

reconciled with the data available on the diversity of nuclear and chromatin-attached protein kinase functions (40, 41).

The repression of the transcriptional activity of BRs on chromosome IV by DRB is accompanied by a regression of puff structure (13) and a reformation of thick chromosome fiber (1). This means that the impairment of a transcriptional event (initiation) (13, 16) is coupled to a major rearrangement (recondensation) in the structure of chromatin (1). These observations in *Chironomus* cells along with the reports from other studies of correlation between phosphorylation of nonhistone proteins and gene activation (29, 31) make it conceivable that the inhibited phosphorylation of the nonhistone proteins, especially the M_r = 42,000 phosphopeptide, reflects the primary action of DRB. The unphosphorylated form of the protein may then mediate or trigger interferences with the function of initiating RNA polymerase II molecules. The stimulation of the phosphorylation of histones H2A and H4, which is somewhat delayed compared with the inhibitory effect on phosphorylation of nonhistone proteins (18), could then be an expression of, or a promoting factor for, reformation of the thick chromosome fiber. The latter possibility receives experimental support from a work dealing with histone phosphorylation which indicates a relationship between chromatin condensation and enhancement of H2A phosphorylation (32).

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