Phosphorylation of Rat C6 Glioma Cell DNA-dependent RNA Polymerase II in Vivo

IDENTIFICATION OF PHOSPHORYLATED SUBUNITS AND MODULATION OF PHOSPHORYLATION BY ISOPROTERENOL AND N\(^{6}\),O\(^{2'}\)-DIBUTYRYL CYCLIC AMP

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Evidence is presented that isoproterenol treatment of rat C6 glioma cells, under conditions that increase glioma cell cAMP levels, causes the phosphorylative modification of several RNA polymerase II subunits. RNA polymerase II in control and isoproterenol-stimulated \(^{32P}\)-labeled confluent glioma cells was immunoprecipitated from ribonuclease-treated nuclear extracts with hen anti-calf RNA polymerase II antiserum conjugated to Sepharose. The immunoprecipitated RNA polymerase II was analyzed for \(^{32P}\)-labeled subunits by electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Using this technique, we have shown that isoproterenol causes a time-dependent increase of phosphate incorporation into RNA polymerase II subunits of 214,000, 180,000, 140,000, 35,000, 28,000, and 16,500 daltons. Phosphate incorporation occurred exclusively on serine in all of the six subunits. About 0.5–2 mol of phosphate/mol of RNA polymerase II subunit were incorporated.

Dibutyryl cAMP (10\(^{-3}\) M) mimics the stimulatory action of isoproterenol and mediates increased phosphate incorporation into the six subunits. (RS)-propranolol (10\(^{-4}\) M) prevents the isoproterenol-mediated phosphorylative changes.

These data indicate that isoproterenol, via cAMP, mediates a transient structural modification of RNA polymerase II subunits in rat C6 glioma cells which may possibly lead to a modulation of RNA polymerase II function(s).

Evidence has accumulated suggesting that covalent phosphorylative modification of enzymes and other regulatory proteins represents a fundamental ubiquitous regulatory mechanism in eukaryotes (1, 2). Among cellular organelles, the nucleus contains the highest concentration of phosphorylated proteins (3, 4) whose phosphate groups are of relatively short half-life and are rapidly turning over. Phosphorylation of selected nuclear proteins has been implicated in the regulation of gene activity primarily on the basis of a temporal correlation between activation of nuclear protein kinase(s) and a concomitant elevation of the synthesis of several RNA species (for review see Ref. 5).

The possibility of a functional regulation of RNA polymerase II via phosphorylation/dephosphorylation has been investigated by several laboratories. At the structural level, RNA polymerase II subunits become phosphorylated in vitro by homologous AMP-dependent, as well as cyclic AMP-independent protein kinases (6–13). RNA polymerase II subunits which become phosphorylated in vitro may also undergo phosphorylation in vivo in yeast (6, 13, 14), plant (15), and mammalian cells (10, 16).

Based on the established regulatory function of phosphorylated proteins in many biological systems, phosphorylative modification of RNA polymerase II can reasonably be expected to be involved in regulating certain functions of the polymerase. However, attempts to demonstrate a correlation between phosphorylation/dephosphorylation and modulation of polymerase function(s) have met with variable success (6–8, 11, 12, 17–19). Thus, the question whether phosphorylation of RNA polymerase II subunit(s) represents a post-translational modification of constitutive nature or, alternatively, serves a regulatory role and modulates RNA polymerase functions remains unsolved.

Rat C6 glioma cells respond to catecholamine and cyclic AMP stimulation by rapidly modulating lactate dehydrogenase A subunit mRNA transcription by RNA polymerase II (20). Therefore, this cell system provides a convenient model to test the effects of RNA polymerase II phosphorylation/dephosphorylation on RNA polymerase function specifically during the period of lactate dehydrogenase mRNA synthesis. As an initial step towards this goal, we have investigated whether RNA polymerase II subunits become selectively phosphorylated after catecholamine treatment of glioma cells.

EXPERIMENTAL PROCEDURES

RESULTS

Characterization of Hen Anti-calf RNA Polymerase II Antiserum—The specificity of the antiserum was established by immunochemical analysis, including Ouchterlony double-diffusion analysis (31), immunoprecipitation analysis, and immunological characterization:

1 The abbreviations used are: cAMP or cyclic AMP, adenosine 3',5'-monophosphate; dibutyryl cAMP, N\(^{6}\),O\(^{2'}\)-dibutyryl adenosine 3',5'-monophosphate; SDS, sodium dodecyl sulfate; PMSF, phenylmethylsulfonyl fluoride; NaCl/Pi, phosphate-buffered saline.

2 Portions of this paper (including "Experimental Procedures" and Figs. 1–3) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1217, cite the authors, and include a check or money order for $5.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
munotitration. Ouchterlony double-diffusion analysis using rabbit anti-hen IgG as secondary antibody showed that the interaction of serum from immunized hens with highly purified calf thymus RNA polymerase II activity as well as with nuclear extracts from nonstimulated and stimulated C6 glioma cells produced a single continuous precipitin line. In contrast, no precipitin band was observed with preimmune serum (results not shown).

Since a preliminary assay showed no detectable inhibitory effect of the hen antiserum on RNA polymerase II activity, rabbit anti-hen IgG was used as secondary antibody to precipitate the soluble primary immunocomplex. After removal of the immunoprecipitate by centrifugation, the supernatant fraction was assayed for residual polymerase activity. As shown in Fig. 1, calf thymus as well as glioma cell RNA polymerase II activity progressively declined after addition of increasing amounts of hen antiserum, indicating that the inhibition of polymerase activity was due to a specific antibody-antigen interaction.

As an additional evaluation of the reactivity and specificity of the hen antiserum, immunoprecipitation of $^{125}$I-labeled calf thymus RNA polymerase II was performed with hen antiserum and preimmune serum. The immunocomplexes were precipitated with rabbit anti-hen IgG and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The immunoprecipitate obtained after incubation of hen antiserum shows a distinct labeling pattern (Fig. 2B, scan I), and six $^{125}$I-labeled polymerase subunits can be identified. In contrast, the immunocomplex formed with preimmune serum and $^{125}$I-labeled RNA polymerase shows no such distinct labeling pattern (Fig. 2B, scan II). The autoradiogram shows a nonpolymerase peptide of an apparent $M_t = 85,000$ which coprecipitated with hen antiserum, indicating that this peptide is tightly associated with calf thymus RNA polymerase II and coprecipitates with the antigen.

The titer of the hen antiserum was determined by incubation of $^{125}$I-labeled calf thymus RNA polymerase II with varying amounts of hen antiserum. Hen preimmune serum was added as supplement to the hen antisera to keep the total amount of hen immunoglobulins constant (47.5 mg/assay) throughout the assays. As shown in Fig. 3, the amount of antigen precipitated depended on the antisera concentration. Half-maximal binding of $^{125}$I-labeled RNA polymerase II was at an antiserum dilution of 1:200, which was equivalent to 0.475 mg of hen immunoglobulins and which corresponded to a ratio of $^{125}$I-labeled antigen to hen antibody of 1:44 (w/w). At 70% of the maximal precipitation, the amounts of labeled antigen and hen antibody were 10.8 ng and 2.1 μg, respectively.

**Effect of Isoproterenol on RNA Polymerase II Subunit Phosphorylation**—To investigate the phosphorylation of RNA polymerase II subunits, it appeared necessary to immunoprecipitate RNA polymerase II from the nuclear protein extract and with a minimum of experimental manipulation in order to avoid artifactual dephosphorylation and proteolysis which might occur despite the presence of inhibitors of protease and phosphatase. Glioma cells were prelabeled with Na$_2$H$^3$P0$_4$, in phosphate-free medium. Stimulation of cells with isoproterenol in the presence of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine was carried out for 1 h. RNA polymerase II was then immunoprecipitated from the nuclear protein extract with chicken anti-calf RNA polymerase II antiserum and rabbit anti-hen IgG. The immunoprecipitate was subjected to SDS-polyacrylamide gel electrophoresis and autoradiography (Fig. 4). Despite the relatively high background density, the phosphorylation of a peptide correponding to the 214-kDa subunit of RNA polymerase II was markedly enhanced in isoproterenol-stimulated cells (Fig. 4, lane D), but not in unstimulated cells (Fig. 4, lane B) or in samples precipitated with preimmune hen serum (Fig. 4, lanes A and C).

Due to the coprecipitation of large amounts of nonspecific radioactivity, it became necessary to partially purify $^{32}$P-labeled RNA polymerase II prior to immunoprecipitation. Removal of nonspecific $^{32}$P was attempted by ribonuclease treatment of nuclear extracts followed by ammonium sulfate precipitation and heparin-Sepharose chromatography. $^{3}$ RNA polymerase II was then isolated with hen antibody-conjugated Sepharose beads. Using this modified experimental approach, the effect of isoproterenol on RNA polymerase II phosphorylation was investigated. In addition to the 214-kDa subunit, increased isoproterenol-mediated $[^{32}P]phosphate incorporation into five subunits of smaller size occurred, e.g. the 180-, 140-, 135-, 28-, and 16.5-kDa subunits (Fig. 5). Under the

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3 After heparin-Sepharose chromatography, RNA polymerase II preparations from control and isoproterenol-stimulated cells exhibited similar specific activities as measured in a conventional polymerase assay (8). Recoveries of RNA polymerase II at this stage were comparable between stimulated and unstimulated cells.
cells was carried with 10 μCi of the S-h labeling period. After the addition of unlabeled glioma cells, RNA polymerase II was then precipitated with ammonium sulfate and subjected to heparin-Sepharose column chromatography as described under "Experimental Procedures." Eluted RNA polymerase was pooled and adsorbed with hen antibody-conjugated Sepharose beads. Adsorbed 32P-labeled RNA polymerase II was dissolved in SDS-sample buffer and applied onto a polyacrylamide gel consisting of 7.5% acrylamide in the upper half (5.5 cm) and 12.5% acrylamide in the lower half (5.5 cm) of the gel. Electrophoresis was carried out at 110 V for 6 h at room temperature. Radioactivity was identified by autoradiography of the gel. Immunoprecipitated RNA polymerase from mock-stimulated glioma cells; B, RNA polymerase II from glioma cells stimulated for 30 min.

In order to minimize artificial experimental variations between stimulated and unstimulated cells, we additionally employed a double-isotope labeling method to identify the phosphorylated subunits. Na2H3'P04 and Na32P04 were used to prelabel the intracellular ATP pools. Mock-stimulated control cells were prelabeled with Na2H3'P04, whereas cells to be stimulated were prelabeled with Na32P04. After isoproterenol stimulation, 32P-labeled stimulated cells were combined with the 32P-labeled unstimulated cells. 32P- and 33P-labeled RNA polymerase II was partially purified from the RNase-treated nuclear extracts and immunoprecipitated. The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and 32P/33P radioactivity was determined in sliced gel sections. The results are illustrated in Fig. 6. The amount of 32P radioactivity/gel slice did not vary appreciably throughout the gel. However, 32P radioactivity increased markedly after stimulation in gel slices, corresponding to the location of RNA polymerase II subunits 214, 180, 140, 35, 28, and 16.5 kDa. The increase of [32P]phosphate incorporation into the subunits after isoproterenol stimulation can also be assessed by comparing in each gel slice the ratio of counts/min 32P versus disintegrations/min 33P incorporated (see Fig. 7). This method of analysis confirms the selective incorporation of phosphate into the six polymerase subunits after isoproterenol stimulation. In about 30% of the experiments, the 240-kDa subunit was also identified as phosphate acceptor.

The 32P/33P ratios of each subunit were determined as a function of stimulation time and are plotted in Fig. 8. Isoproterenol-mediated phosphorylation of the 214- and 35-kDa subunits occurred already after a 5-min stimulation time. Maximal phosphorylation levels were reached after a 15-min stimulation period. The level of phosphorylation, as indicated by the 32P/33P ratios, had declined again after a 2-h stimulation period, but remained still slightly elevated in the 214-kDa subunit.
Effect of Dibutyryl Cyclic AMP and (RS)-Propranolol on RNA Polymerase II Phosphorylation—Isoproterenol-mediated phosphorylation was prevented in the presence of (RS)-propranolol, indicating that the stimulatory effect of the β-adrenergic agonist was successfully inhibited by the antagonist (results not shown). To test whether dibutyryl cAMP mimics the action of isoproterenol, glioma cells were stimulated with the cAMP analogue. Phosphate incorporation into RNA polymerase II subunits was assessed by the double 32P and 33P isotope labeling method. The results are shown in Table I. Similar to the action of isoproterenol, dibutyryl cAMP caused a significant increase of [32P]phosphate incorporation into six RNA polymerase subunits. Addition of sodium butyrate (at 10^{-3} M) had no effect on [32P]phosphate incorporation (data not shown).

Effect of Cycloheximide on RNA Polymerase II Phosphorylation—The protein synthesis inhibitor cycloheximide (25 μg/ml) was added to glioma cell cultures 30 min before a 45-min stimulation period with isoproterenol. Under the conditions of the experiment, the presence of cycloheximide blocked [35S]methionine incorporation into total protein but did not affect the isoproterenol-mediated phosphorylation of the six subunits (data not shown). Based on these findings, de novo protein synthesis is not required for isoproterenol to achieve its effect, indicating that phosphorylation of RNA polymerase II subunits is a post-translational modification.

Analysis of Phosphoamino Acids—To identify the phosphate acceptor amino acid, [32P]RNA polymerase II was isolated by immunoabsorption to hen antibody-conjugated Sepharose beads. Electrophoretically separated subunits were subjected to partial acid hydrolysis, and [32P]phosphoamino acids were separated by high voltage paper electrophoresis. As shown in Fig. 9 for the 214-kDa subunit, only [32P]phosphothreonine was identified in the 32P-labeled subunit. Identical results were obtained for the 180-, 140-, 35-, 28-, and 16.5-kDa subunits. [32P]Phosphothreonine and [32P]phosphotyrosine were not detected. The [32P]phosphate of the 32P-labeled subunits was acid-stable, and no acid-labile [32P]phosphate was detected (results not shown).

DISCUSSION

Structural and functional modification of nonhistone chromosomal proteins through phosphorylation/dephosphorylation has been suggested as one of the epigenetic mechanisms involved in the regulation of gene expression in eukaryotes. This hypothesis is based on established temporal correlations between phosphorylation/dephosphorylation and gene activation (5, 32). The possibility of a regulation of RNA polymerase II function via phosphorylation/dephosphorylation has been considered by a number of researchers. However, studies attempting to correlate phosphorylative modification and modulation of polymerase function have not yielded uniform results. While some investigators reported that in vitro phosphorylation of RNA polymerase II leads to an increase in
were treated with 10-laheled control cells were mock stimulated. Incubation was continued with an identical number of "P-labeled stimulated cells. Polyacrylamide gel electrophoresis were carried out as described in the legend of Fig. 6. The gel was sliced into 2-mm gel sections and the amount of "P and "P in each gel slice were determined. The values represent the average of four separate experiments.

Isolation of nuclei, partial purification, immunoabsorption, and SDS-polyacrylamide gel electrophoresis were carried out as described under "Comparison of the effects of isoproterenol and dibutyryl cAMP stimulation of RNA polymerase II subunits".

RNA polymerase II Subunit Phosphorylation

| Subunit | Treatment | Radioactivity in subunit | Ratio | 
|---------|-----------|-------------------------|-------|
|         |           | cpm | dpm | cpm/dpm |         |
| 214 kDa | Control   | 125.1 | 81.2 | 1.5 |
|         | Isoproterenol | 492.3 | 107.3 | 4.6 |
|         | Dibutyryl cAMP | 610.7 | 148.5 | 4.1 |
| 180 kDa | Control   | 122.0 | 71.0 | 1.7 |
|         | Isoproterenol | 408.6 | 133.2 | 3.1 |
|         | Dibutyryl cAMP | 600.2 | 135.4 | 4.4 |
| 140 kDa | Control   | 107.0 | 75.4 | 1.4 |
|         | Isoproterenol | 429.2 | 141.5 | 3.0 |
|         | Dibutyryl cAMP | 540.0 | 167.3 | 3.3 |
| 35 kDa  | Control   | 126.0 | 76.9 | 1.6 |
|         | Isoproterenol | 1,012.2 | 296.7 | 3.4 |
|         | Dibutyryl cAMP | 1,620.7 | 444.4 | 3.6 |
| 28 kDa  | Control   | 130.1 | 104.2 | 1.3 |
|         | Isoproterenol | 326.3 | 133.0 | 2.5 |
|         | Dibutyryl cAMP | 425.1 | 106.2 | 4.0 |
| 16.3 kDa | Control | 60.1 | 53.7 | 1.1 |
|         | Isoproterenol | 137.4 | 69.1 | 1.5 |
|         | Dibutyryl cAMP | 255.7 | 63.7 | 4.0 |

Enzymatic activity (5, 8, 18, 19) or RNA chain initiation (11), others failed to find such correlations (6, 10, 14).

So far, no studies on RNA polymerase II phosphorylation have been carried out at times of physiological transition such as development or hormonal stimulation leading to altered gene expression and synthesis of specific gene products. Studies in such experimental systems would allow examination of a possible correlation between RNA polymerase II phosphorylation and function. The experimental system used by us is ideally suited for such studies. However, in order to determine if glioma cell RNA polymerase II undergoes phosphorylative modification in vivo after isoproterenol stimulation, it was necessary to develop a procedure for the purification of RNA polymerase II from small quantities of cells. The procedure is based on the fact that antiserum against calf thymus RNA polymerase II cross-reacts efficiently with RNA polymerase II of other mammalian species (25, 33). We have applied an immunoprecipitation procedure which avoided a lengthy purification and allowed the isolation and identification of RNA polymerase II from a relatively small number of cells in a reproducible fashion. Application of this procedure resulted in the isolation of immunoprecipitated glioma cell RNA polymerase II which had polypeptide subunits of 214, 180, 140, 165.5 kDa. In several experiments, a 240-kDa subunit was also identified. This subunit structure is similar to that of calf thymus (220, 180, 140, 34, 25, and 16.5 kDa) and rat liver (220, 214, 180, 140, 34, 25, and 16.5 kDa) RNA polymerase II described by Kedinger et al. (34). Our attempts to selectively immunoprecipitate [32P]RNA polymerase II directly from nuclear extracts were not successful because the coprecipitation of nonspecific [32P]-labeled material caused a high background in the autoradiographs and prevented identification of several [32P]-labeled subunits. The contaminants may be minor but are probably very highly labeled. Of various purification procedures evaluated, it appeared to be important to treat [32P]-labeled nuclear extracts with ribonuclease. This led to an efficient removal of contaminants (probably mostly [32P]RNA).

To investigate the in vivo sites of RNA polymerase II phosphorylation, the enzyme was isolated from confluent unstimulated and stimulated glioma cells that had been labeled with isotope for several hours. RNA polymerase II from unstimulated cells contained only low levels of [32P], and prolonged exposures (longer than the ones used here) were required to detect this level of radioactivity by autoradiography. However, isoproterenol treatment of glioma cells led to a marked incorporation of [32P]phosphate into six RNA polymerase II subunits in a time-dependent fashion. Chemical analysis of [32P]polymerase subunits indicated that the incorporation of phosphorus occurred primarily on serine in all six subunits. This is in agreement with our previous studies which identified serine as the principal phosphate acceptor of calf thymus RNA polymerase II in vitro (8). The temporal pattern of phosphate addition was similar for all six subunits, but whereas phosphorylation of the 214- and 35-kDa subunits remained slightly elevated 2 h after stimulation, the level of phosphorylation of the other four subunits had declined to control levels by that time.

In cell culture, the stoichiometry of the phosphate incorporation into the individual RNA polymerase subunits is difficult to determine with accuracy, but it is possible to obtain approximate estimated values. We have previously shown that glioma cell cultures equilibrated with Na2H[32P]PO4 will incorporate [32P] into [32P]ATP linearly up to 3 h of labeling time to reach a specific activity of about 400 cpm of [32P]/pmol of ATP. After 2 h, the specific activity of [32P]ATP remained

![Fig. 9. Analysis of [32P]phosphoamino acids by paper high-voltage electrophoresis. Cells were labeled with Na2H[32P]PO4 and stimulated for 0, 5, and 30 min with 10 μM isoproterenol as described in the legend of Fig. 7. RNA polymerase was subjected to SDS-gel electrophoresis. Partial acid hydrolysates of gel slices containing the 214-kDa subunit were fractionated by paper electrophoresis at pH 2.4 (1% formic acid, 4% acetic acid; 500 V; 16 h) as described under "Experimental Procedures." [32P]Phosphoamino acids were visualized by autoradiography and identified by comigration of authentic standards. Lane A, control cells; lane B, cells stimulated for 5 min; lane C, cells stimulated for 30 min.](image-url)
constant for an additional 4-h period and was not altered by isoproterenol or dibutyryl cAMP treatment of the cells (24). Using this experimentally determined value, it can be estimated that there were about two phosphate groups incorporated per 140-kDa subunit. The relative disparity in the labeling of the individual subunits (Fig. 5) suggests that the 214-, 186-, 28-, and 16.5-kDa subunits incorporate one phosphate group/subunit and the 35-kDa subunit only 0.5 phosphate group/subunit.

Several lines of evidence indicate a post-translational mechanism of RNA polymerase II phosphorylation. First, inhibition of protein synthesis by cycloheximide had no discernible effect on the isoproterenol-mediated phosphorylations. The major question of whether isoproterenol-mediated phosphorylation of RNA polymerase II has any functional significance in the C6 glioma cell remains unsettled. The rat C6 glioma cell responds to β-adrenergic agonists with an activation of its adenyl cyclase leading to a rapid transient rise of cAMP levels (35) and activation of nuclear cAMP-dependent protein kinase (36, 37). This is followed by an increased rate of transcription of lactate dehydrogenase A subunit mRNA (20) and increased activity of a number of enzymes such as cAMP phosphodiesterase (38), ornithine decarboxylase (39), 2′, 3′-cAMP-3′ phosphohydrolase (40, 41), and phosphorylase α (42). Catecholamines or dibutyryl cAMP also potentiate the glucocorticoid induction of glycolate phosphate dehydrogenase (43) and increase the levels of S-100 protein in nonconfluent cell cultures (44) and of β-nerve growth factor (45, 46).

It is of interest that the phosphorylation of glioma cell RNA polymerase II subunits occurs concomitantly with the phosphorylation of several other chromosomal (24, 47, 48) and cytoplasmic (49) proteins. Thus, there is a strong physiological correlation between the pattern of phosphorylation of several glioma cell proteins and altered gene expression after catecholamine stimulation. It is tempting, therefore, to assume that a phosphorylative and functional modification of RNA polymerase II may play a role in the altered pattern of gene expression in glioma cells. Having demonstrated a catecholamine- and cAMP-mediated structural modification of RNA polymerase II, it is now necessary to identify the physiological role of the phosphorylation. The introduction of negative charges onto the RNA polymerase II molecule could modulate inter- as well as intramolecular interactions. Therefore, it is necessary to measure the effect of RNA polymerase phosphorylation on several parameters such as its binding affinity for specific regulatory gene sequences, correct initiation and termination of transcription, and enzyme half-life.

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RNA Polymerase II Subunit Phosphorylation

EXPERIMENTAL PROCEDURES

Materials - Tissue culture media were purchased from Grand Island Biological Company, Corn Harbour, New Brunswick, Canada, and Sigma Chemical Company, St. Louis, Missouri. Antibiotics were obtained from Sigma Chemical Company unless indicated otherwise. The following anti-RNA polymerase antibodies were used: MAb 11 (anti-subunit 11), C6 (anti-subunit 6), and 18 (anti-subunit 18). For immunochemical analyses, the antiserum to anti-RNA polymerase was used. All other biochemical reagents were purchased from Sigma Chemical Company. The following antiserum was used: anti-RNA polymerase 11 (anti-subunit 11). All other biochemical reagents were purchased from Sigma Chemical Company. The following antiserum was used: anti-RNA polymerase 11 (anti-subunit 11). All other biochemical reagents were purchased from Sigma Chemical Company.

Preparation of Cell lysates - RNA polymerase II was isolated from cell lysates by the procedure of Schiavo et al. [29] using MAb 11 and C6 antibodies. The final purification steps were performed by glycerol density gradient ultracentrifugation. The purity of protein A-Sepharose was determined by SDS-PAGE and Western blot analysis. The purity of RNA polymerase II was determined by Western blot analysis. The purity of the final product was confirmed by SDS-PAGE and Western blot analysis.

Preparation of Nuclear Extracts - After the appropriate incubation and stimulation of cells, the nuclear extract was prepared by the method of Schiavo et al. [29]. The nuclear extract was then subjected to immunoprecipitation and Western blot analysis. The purity of the nuclear extract was confirmed by SDS-PAGE and Western blot analysis.

Preparation of Antiserum - hen antiserum was prepared by immunizing the hen with RNA polymerase II. The antiserum was then subjected to immunoprecipitation and Western blot analysis. The purity of the antiserum was confirmed by SDS-PAGE and Western blot analysis.

Preparation of Antibodies - RNA polymerase II was isolated from cell lysates by the procedure of Schiavo et al. [29] using MAb 11 and C6 antibodies. The final purification steps were performed by glycerol density gradient ultracentrifugation. The purity of protein A-Sepharose was determined by SDS-PAGE and Western blot analysis. The purity of RNA polymerase II was determined by Western blot analysis. The purity of the final product was confirmed by SDS-PAGE and Western blot analysis.

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