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# Discrimination between RNAP IIA and IIO in Preinitiation Complex Assembly and Tyrosine Phosphorylation of the Carboxy Terminal Domain

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**Abstract :** Multiple phosphorylation of the carboxy-terminal domain (CTD) of the largest subunit in RNA polymerase II (RNAP II) is thought to play an important role in the transcription cycle. The preinitiation complex in a partially purified complete transcription system suggested that RNA polymerase IIA containing unphosphorylated CTD is involved in complex assembly, whereas RNA polymerase IIO containing Ser and Thr phosphorylated CTD is not involved in preinitiation complex assembly. Recently a minimal transcription system was developed which requires chemically defined minimal components for its transcription: TBP, TFIIB, TFIIF, RNAP II and a supercoiled adenovirus-2 major late promoter (Ad-2 MLP). It would be using interesting to determine the consequence of CTD phosphorylation on preinitiation complex formation using the minimal transcription system. Contrary to the results from the partially purified complete transcription system, both RNA polymerase IIA and IIO are equally recruited in the preinitiation complex formation. The discrepancy may result from the two different assays used to determine complex formation, the use of chemically undefined complete and defined minimal transcription systems. This implicates that some factors in the complete transcription system are involved in the distinction between RNAP IIA and IIO in complex assembly. In addition multiple tyrosine phosphorylation of the CTD of RNAP II was prepared with c-Abl kinase and its recruiting ability in the preinitiation complex was examined. Compare with Ser and Thr phosphorylated RNAP IIO, Tyr phosphorylated RNAP IIOy forms a stable preinitiation complex in both the minimal and complete transcription systems. Based on these results, it seems that tyrosine phosphorylation of the CTD is important in the transcription cycle on the special subset of class-II promoter or has a different role in the transcription process.

**Key words :** Ad-2 MLP, c-Abl kinase, carboxy-terminal domain, IIO and IIOy, RNA polymerase IIA

RNA polymerase II (RNAP II) is a large multisubuint enzyme composed of two large subunits with a molecular weight of greater than 100 kDa and 8-10 smaller subunits (Sawadogo and Sentenac, 1990). The largest subunit of RNA polymerase II contains at its carboxyl terminus a highly repetitive domain consisting of tandem repeats of the 7-amino acid consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser, designated the carboxy-terminal domain (CTD) (Corden, 1990; Young, 1991; Dahmus and Dynan; 1992). The heptapeptide repeat is present in 52 copies in mammals, 42 copies in Drosophila, and 26~27 copies in yeast (Corden, 1990). Each repeat contains multiple potential phosphorylation sites, and indeed RNAP II can be found in both the nonphosphorylated and highly phosphorylated forms *in vivo* and *in vitro* (Cardena and Dahmus, 1987). The unphosphorylated form of the largest subunit is designated IIa having an apparent molecular weight of 214,000, whereas the phosphorylated form of this subunit is designated IIo having an apparent molecular weight of 240,000 (Kim and Dahmus, 1988). The enzymes containing these subunits are designated RNAP IIA and RNAP IIO, respectively (Kim and Dahmus, 1986).

Genetic analysis in yeast, *Drosophila* and mouse have shown that at least half of the number of repeats normally present are essential for cell viability (Bartolomei *et al.*, 1988; Nonet *et al.*, 1987). *In vitro* transcription studies on variety of promoters have shown that the CTD is essential for transcription from some promoters and not from others (Buermeyer *et al.*, 1992). Furthermore, the CTD has been shown to play a critical role in the recruitment of RNAP II to the dihydrofolate reductase promoter (Kang and Dahmus, 1993). Phosphorylation of the CTD has been proposed to trigger the transition from complex assembly to transcript elongation

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(Dahmus and Dynan, 1992). An analysis of the state of CTD phosphorylation as a function of the position of RNAP II in the transcription cycle has lead to the proposal that each cycle of transcription by RNAP II is associated with the reversible phosphorylation of the CTD (Payne *et al.*, 1989). Considerable evidence derived from the complete transcription system using reconstituted cell transcription extract supports the idea that RNAP IIA, the unphosphorylated form of RNAP II, interacts with the promoter to form a stable preinitiation complex (Chesnut *et al.*, 1992).

Recently a minimal transcription system was developed which requires chemically defined minimal components for its transcription: TBP, TFIIB, RNAPII and a negatively supercoiled immunoglobulin heavy chain promoter (Parvin and Sharp, 1993). Initiation at the adenovirus-2 major late promoters (Ad-2 MLP) was observed with the minimal transcription system containing TBP, TFIIB, TFIIF, RNAPII and a negatively supercoiled Ad-2 MLP (Parvin and Sharp, 1993). On the other hand, transcription from linear DNA templates requires in addition TFIIF, TFIIE, TFIIH, and a fraction containing TFIIA and TFIIJ. It seems that the free energy of supercoiling promotes the formation of an open complex for initiation of transcription by a minimal set of transcription factors. Therefore it is interesting to determine the consequence of CTD phosphorylation on preinitiation complex formation with the minimal transcription system.

A number of kinases that phosphorylate the CTD on serine and threonine residues have been identified (Dahmus and Dynan, 1992; Feaver *et al.*, 1991; Lu *et al.*, 1992; Serizawa *et al.*, 1992; Payne and Dahmus, 1993; Peterson *et al.*, 1992). The multiplicity of kinases suggested that the interaction between the CTD and preinitiation complex may be regulated by different kinases depending on the context of the promoters. Recently Baskaran *et al.* (1993) reported that c-Abl kinase could incorporate phosphates on tyrosine residues of the CTD *in vivo*. Also, multiple tyrosine phosphorylation of the CTD with c-Abl kinase was observed in vitro assays. C-Src tyrosine kinase does not phosphorylate the CTD under identical *in vitro* conditions indicating that the CTD is not a general substrate for all tyrosine kinases. The c-abl protooncogene encode a protein tyrosine kinase found both in the cytoplasm and in the nucleus. These results suggest that tyrosine phosphorylation of the CTD may have a role in the preinitiation complex formation during the transcription process. The tyrosine residues of each heptapeptide repeat in the CTD bind to the minor groove of DNA and phosphorylation of tyrosine loosens that binding (Huang *et al.*, 1994). Together with the modulation of DNA binding the phosphotyrosine may serve as binding sites for nuclear SH2 proteins allowing the formation of specific CTD-protein complexes. SH-2 domain is generally found in all the proteins involved in signal transduction pathways (Pawson and Gish, 1992). Especially it is interesting to find that the subunits of interferon-regulated transcription factor ISGF3 contains an SH2 domain (Fu, 1992). In this regard it is very interesting to elucidate the biochemical role of tyrosine phosphorylation of the CTD. Therefore purpose of the study presented here is to examine directly the effect of CTD phosphorylation on the ability of RNAP II to interact with the Ad-2 MLP and form a complex assembly in the minimal transcription system. Also the consequence of tyrosine phosphorylation of the CTD on preinitiation complex formation was determined in the minimal and complete transcription systems.

## Materials and Methods

### Materials and plasmids

Plasmids, pGEM4-c-*abl* (type IV) containing the c-*abl* c-DNA in the pGEM4 vector, pACSG-HisNT-A (Baculovirus expression vector) and anti-Abl (8E9) were kindly provided from Jean Wang (University of California, San Diego). Radiolabeled ribonucleotide [$\gamma$-$^{32}$P] ATP (3000 Ci/mmol) was obtained from Amersham Corp. Ultrapure nucleotides were purchased from Pharmacia LKB Biotechnology Inc. Ni-NTA agarose was obtained from Qiagen.

### Preparation of $^{32}$P-labeled substrates

RNA polymerase II was purified from calf thymus by the procedure of Hodo and Blatti (1977) with the modifications described by Kang and Dahmus (1993). Casein kinase II was purified as described by Payne *et al.* (1989). CTD kinase was recovered from the DE0.15 fraction (HeLa cell extract was eluted from DEAE-5PW with 0.25 M KCl.) The fraction containing the highest activity was precipitated in the presence of 45% saturated ammonium sulfate and dissolved in and dialyzed against 20 mM Tris-HCl, pH 7.9, 20 mM KCl, 7 mM $MgCl_2$, 0.5 mM DTT, 0.05 mM EDTA, 0.012% Triton X-100, and 10% glycerol.

The largest subunit of mammalian RNAP II was labeled by phosphorylation of the most C-terminal serine residue by incubation with casein kinase II in the presence of [$\gamma$-$^{32}$P] ATP (Chesnut *et al.*, 1992). Phosphorylation at this site does not alter the electrophoretic mo- RNAP IIO was prepared by phosphorylation of ATP RNAP IIA with a partially purified CTD kinase and [$\gamma$-$^{32}$P] ATP RNAP IIA with a partially purified CTD kinase and in the presence of excess unlabeled 2 mM ATP (Chesnut *et al.*, 1992). Extensive phosphorylation within the CTD

decreases the electrophoretic mobility of subunit IIa in SDS-PAGE. Consequently, the state of phosphorylation of RNAP II in preinitiation complexes can be determined by direct comparison of the mobility of the largest polymerase subunit in the complex relative to the mobility of marker subunit IIa and IIo (Payne *et al.*, 1989).

**Expression and purification of c-Abl kinase**

In order to purify c-Abl kinase more easily, the gene for $(His)_6$ peptide was tagged to the N-terminus of c-*abl* gene. For this purpose *Eco*RI and *Hind*III fragment (c-*abl* gene) was introduced to Bluescript KS(-). Then *Eco*RI and *Kpn*I fragment of c-*abl* in Bluescript KS(-) was reintroduced to baculovirus transfer vector pACSG-HisNT-A. Recombinant baculovirus was isolated by plaque purification as described by Summers and Smith (1988). For the purification of c-Abl kinase 500 ml suspension culture of Sf21 cell was grown in a simple round flask with a flattened bottom. This 500 ml culture (in a 1000 ml flask with $10^5$ cells per ml) was incubated at 28℃ with mild stirring until the cell density is $4\sim5\times10^5$ per ml (2 days). The culture was inoculated with virus at a multiplicity of infection of 0.1 and incubated at 28℃ for 3~4 days. The cells were harvested and lysed in lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 100 mM Vanadate, 20 μg/ml leupeptin, 20 μg/ml pepstain, 200 μg/ml aprotinin and 100 μg/ml PMSF). After incubation for 40 min at 0~4℃, the lysates were centrifuged for 10 min at 12.000×g. The supernatant was loaded onto a p11 column equilibrated with 25 mM Tris-HCl pH 7.7, 0.1 % Triton X-100. After stepwise-elution of 0.25-1 M NaCl solution, 0.4-0.6 M NaCl fractions were collected. These fractions were dialyzed with nickel column buffer (10 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 0.1 mM EDTA, 50 mM NaCl, 1 mM DTT, 17% glycerol and 0.1 mM PMSF) and loaded into Ni-NTA agarose column. Finally 160 mM imidazole fractions were collected and stored at -80℃ until use.

**Kinase reactions by c-Abl kinase**

The kinase reaction buffer for c-Abl kinase was 5 mM Tris-HCl pH 7.9, 5 mM $MgCl_2$, 1 mM DTT, 5.4% glycerol and 0.007% TritonX-100. $^{32}$P-labeled RNAP IIA (2000~5000 cpm) and 10 μM ATP were added to a purified c-Abl kinase (2~3 μl) in a total volume of 25 μl of kinase buffer. After incubation at 30℃ for 30 min, reactions were stopped by the addition of sample loading buffer and resolved by SDS-PAGE.

**Formation and fractionation of transcription complexes from free RNA polymerase**

Preinitiation complexes were assembled on the Ad-2 MLP with $^{32}$P-labeled RNAP IIA, IIO, or a mixture of RNAPs IIA and IIO in a 3 × transcription reaction buffer (total volume was 60 μl) and incubated for 30 min at 30℃ in the absence of ribonucleotides (Fig. 1). Preinitiation complexes of complete transcription were formed by incubation of 12 μl of TFIIA, 48 μl of DE0.15 (HeLa cell extract was eluted from DEAE-5PW with 0.15 M KCl.), 24 μl of DE0.25 (HeLa cell extract was eluted from DEAE-5PW with 0.25 M KCl.), 3 μl of template, and RNAP IIA or IIO in buffer. Also preinitiation complexes of the minimal transcription reactions were formed by incubation of 5 μl TBP, 5 μl TFIIF, 0.25 μl TFIIB, 5 μl template, and RNAP IIA or IIO. The complexes were fractionated from free RNAP II by chromatography on a 3.5 ml Sepharose CL-4B column equilibrated and eluted with buffer B containing 37.5 mM KCl and 6 mM $MgCl_2$ as described by Carey *et al.*

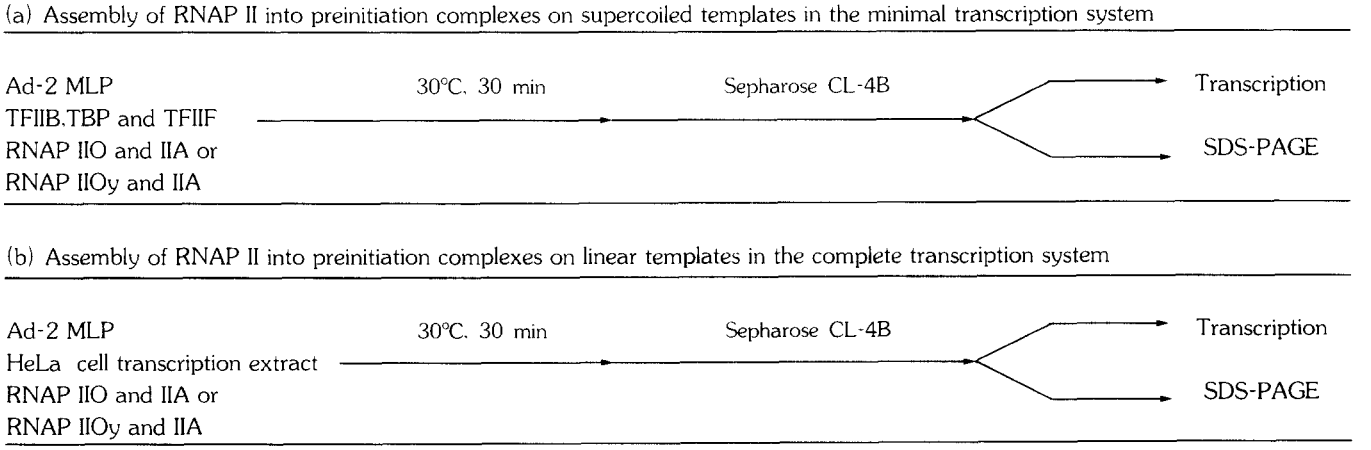


**Fig. 1.** Schematic presentation for preinitiation complex assembly and fractionation. (a) the minimal transcription (b) the complete transcription.

(1986). Fractions of 150 μl were collected. Aliquots of 50 μl were removed, denatured, and run on a 5% polyacrylamide-SDS gel as previously described by Wray et al. (1981), dried, and exposed to x-ray film. Also transcription activities with the complete and minimal transcription reactions were performed with all fractions of Sepharose CL-4B column chromatography as described previously (Chesnut *et al.*, 1992; Tyree *et al.* 1993). The Ad-2 MLP template (position -260 to 560) was prepared as described in Kang and Dahmus (1993). The Ad-2 MLP template gives a 560-nucleotide run-off transcript.

## Results and Discussion

### *In vitro* transcription systems

There are three kinds of *in vitro* transcription systems developed and classified depending on the properties of transcription reaction mixture (Buratowski 1994). At first transcription reaction was carried out by using the partially purified nuclear extracts of cells. This mixture contains many transcription factors. The produced transcript is regulated by specific transcription factors and called activated transcription or undefined complete transcription system. Due to more understanding the transcription process, it is possible to carry out the transcription reaction with only purified general transcription factors and RNA polymerase II. Such transcription is the result of basal activity from the template and called basal or defined reconstituted transcription. Recently transcription reaction from supercoiled template requires only three general transcription factors such as TBP, TFIIB, and TFIIF and RNAP II (Parvin and Sharp, 1993). Due to the potential energy in supercoiled template, open complex formation is more easily formed than on linear template. This transcription requires minimum components and is called minimal transcription.

### Interaction of RNA IIA and IIO with the Ad-2 MLP in the minimal transcription

The CTD modifications that have been demonstrated so far include the phosphorylation of serine and threonine (Cardena and Dahmus, 1987), the phosphorylation of tyrosine (Baskarahan *et al.*, 1993) and the addition of N-acetylglucosamine to serine and threonine (Kelly *et al.*, 1993). All of these modifications occur at multiple sites within the CTD of RNAP II and may be mutually exclusive. Previously the effect of phosphorylation of serine in the CTD on the ability of RNAP II to interact with the Ad-2 MLP was measured directly by determining the amount of RNAP IIA and IIO in preinitiation complex formed in a partially purified complete transcription. In such experiments, it was suggested that

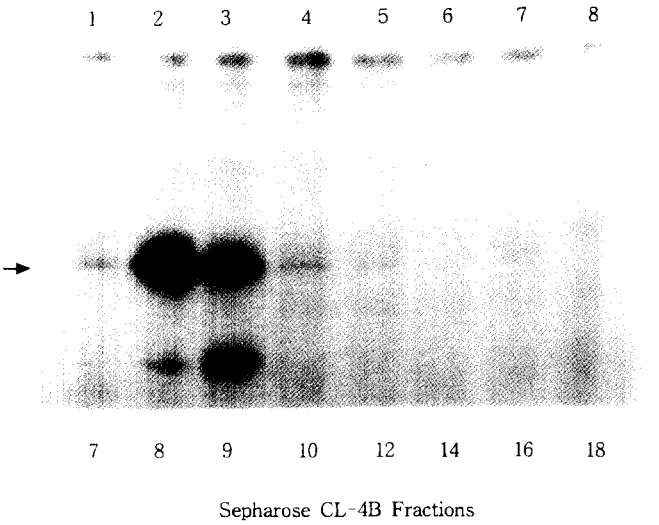


**Fig. 2.** Transcriptional activity with minimal transcription reaction of each fraction of preinitiation complex assembly experiment in the minimal transcription system. Aliquots of each fractions from Sepharose CL-4B columns presented in Fig. 1 were removed and assayed for transcriptional activity as described in Materials and Methods. The numbers below the autoradiogram correspond to specific column fractions.

RNA polymerase IIA is involved in the complex assembly, whereas RNA polymerase IIO is not involved in preinitiation complex assembly (Chesnut *et al.*, 1992).

In order to identify the recruiting preference of RNAP IIA in preinitiation complex assembly, the minimal transcription system was adopted. An equivalent number of units of [$^{32}$P] RNAP IIA and IIO were incubated in the presence of the minimal transcription factors and the Ad-2 MLP (Fig. 1) and preinitiation complexes were fractionated from free RNAP II and other reaction components by chromatography on Sepharose CL-4B. Aliquots of the reactions were removed at the end of preincubation for the minimal transcription reactions. Fig. 2 shows that only excluded fractions (lane 2 and 3) make transcription products. I included fractions (lane 5-8) do not make any transcription products. This means that the excluded fractions containing Ad-2 MLP, RNAP II and several proteins are preinitiation complexes and the included fractions are free RNAP II. The distribution of RNAP II in various column fractions and the state of phosphorylation of the largest subunit were determined by SDS-PAGE. As shown in Fig. 3, both RNAP IIA and IIO are recruited in the preinitiation complexes (excluded fractions, lane 2~4). About 23 and 22% of adding RNAP IIA and IIO are assembled in preinitiation complex (upper panel of Fig. 3). To count the nonspecific assembled complex formations in the absence of Ad-2 MLP or three general transcription factors or both were tried. In these conditions about 4~10% of nonspecific complexes were formed. Although

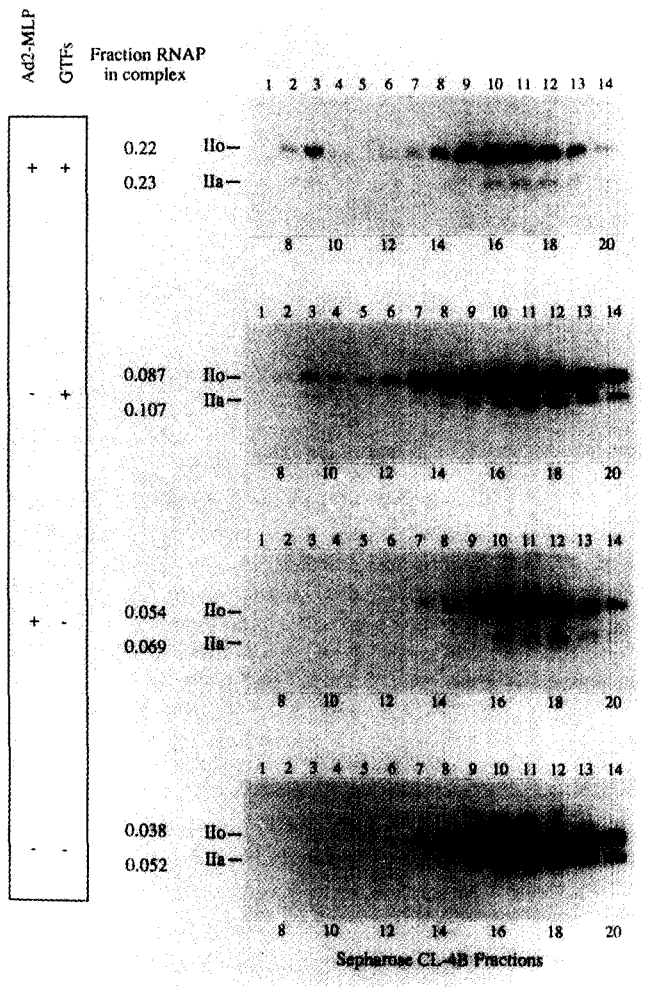


**Fig. 3.** The analysis of preinitiation complexes formed in the presence of RNAP IIA and IIO in the minimal transcription conditions. Preinitiation complexes were formed and analyzed as described in Fig. 1 and Materials and Methods. The amount of label in specific bands were quantitated on a phosphorimager. The fraction of preinitiation complex was calculated as the percent of complex form (the label amount of lane 2~4 divided by the label amount of total lanes, lane 1~14). The numbers below each autoradiogram correspond to specific column fractions.

about 10% of nonspecific complex was made in the absence of Ad-2 MLP and in the presence of general transcription factors (1st middle panel of Fig. 3), fractions of nonspecific complexes made in other conditions (2nd middle and bottom panel of Fig. 3) are lower than that of specific complex (upper panel of Fig. 3). These results showed that no discrimination between RNAP IIA and RNAP IIO was observed in complex assembly and are contradictory to the previous preinitiation complex assembly of the complete transcription system. The observation that *in vitro* transcription of the Ad-2 MLP is inhibited by monoclonal antibody directed against the CTD when transcription is carried out in the presence of Rat-factor T (TFIID) but not in the presence of recombinant TBP suggests that the interaction between the CTD and native TATA factor not TBP may be important in the recruitment of RNAP II to the initiation complex (Conaway *et al.*, 1992). A functional link between the CTD and TFIID is also supported by the finding that SRB2, which binds to TFIID, can suppress the activity of CTD truncation mutants (Thompson *et al.*, 1993). This evidence suggests that TFIID participates in the distinction between RNAP IIA and IIO in complex assembly. To examine the involvement of TFIID in preinitiation complex distinction between RNAP IIA and IIO, purification of TFIID and preinitiation complex formation in the minimal transcription system are under investigation.

**Multiple tyrosine phosphorylation of the CTD with c-Abl kinase**

It has only recently become apparent that the CTD can be phosphorylated on tyrosine (Baskarahan *et al.*, 1993). Previous attempts to identify the *in vivo* sites of phosphorylation in both mammalian cells and yeast established that serine is the primary site of phosphorylation, with some phosphorylation also occurred on threonine. Recent studies, in which phosphotyrosine was recovered from RNAP II immunoprecipitated from HeLa cells, differed from earlier work in that a mixture of phosphatase inhibitors was included to inhibit the activity of tyrosine phosphatases. Furthermore, a nuclear tyrosine kinase encoded by the c-abl proto-oncogene phosphorylates the CTD *in vitro* on approximately 30 sites, resulting in the characteristic mobility shift in SDS-PAGE (Lee, 1995).

To express and purify the c-Abl kinase, the gene for $(His)_6$ peptide was tagged to the N-terminus of c-*abl* gene. The construction of the vector for expressing (His) tagging c-Abl kinase was described previously by Lee (1995). This vector is designed to be expressed under the control of a polyhedrin promoter using the baculovirus system. The identification of c-abl gene expression was done by Western blotting with antibody against c-Abl kinase (data not shown). The kinase was the full length polypeptide as judged by SDS PAGE (120 kDa) and the expression of c-Abl kinase was as much as 5% of the total protein. Purification of c-Abl kinase was done by chromatography on phosphocellulose P11 and Ni-NTA column chromatography which can be monitored by mobility shift assay (Lee, 1995). The labelled RNAP IIa was shifted to the position of RNAP IIy by increasing amounts of c-Abl kinase (Fig. 4). This molecular shift was similar to the shift from RNAP IIa to RNAP IIo by CTD kinase. A nuclear tyrosine kinase encoded by the c-abl protooncogene phosphorylates the

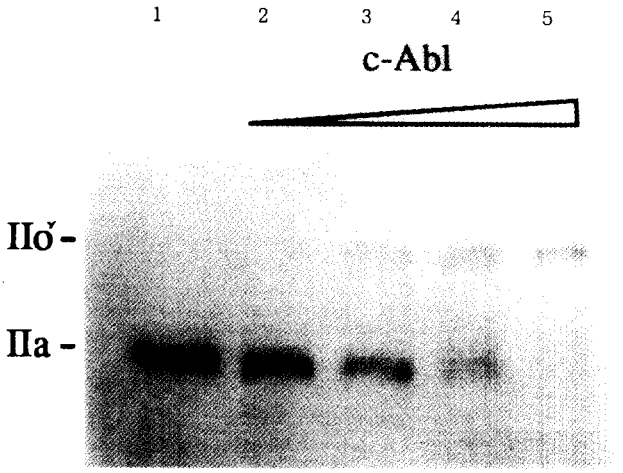


**Fig. 4.** Mobility shift and multiple phosphorylation of the largest subunit of RNA Polymerase II with c-Abl kinase. Labeled RNAP IIA was incubated with ATP and increasing amount of c-Abl kinase. Lanes 1-5 contained 0. 1.2.3 and 4 μl of c-Abl kinase. Using the baculovirus expression system His-tagging c-Abl kinase was expressed and purified with Ni-NTA affinity column.

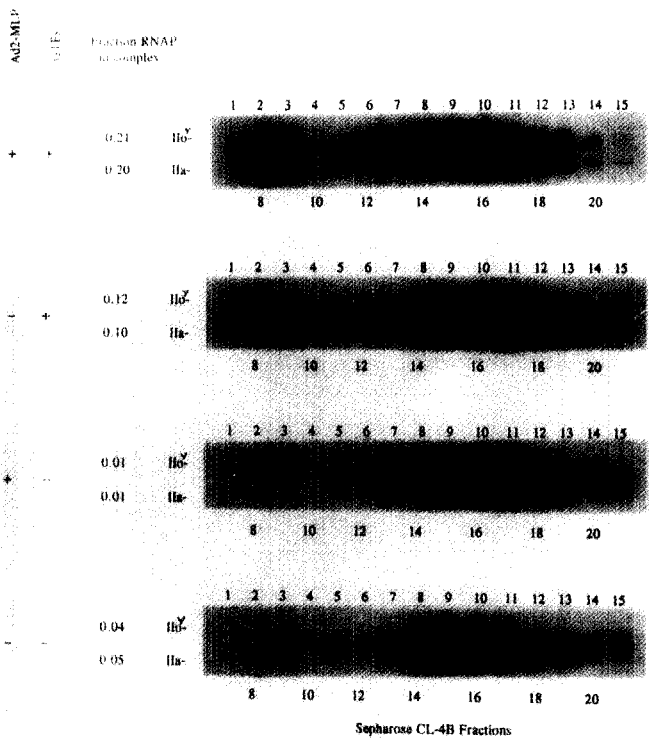


**Fig. 5.** The analysis of preinitiation complexes formed in the presence of RNAP IIA and IIOy (c-abl kinase labeled) in the minimal transcription conditions. Preinitiation complexes were formed and analyzed as described in Fig. 1 and Materials and Methods. The amount of label in specific bands were quantitated on a phosphorimager. The fraction of preinitiation complex was calculated as the percent of complex form (the label amount of lane 2~4 divided by the label amount of total lanes. lane 1~14). The numbers below each autoradiogram correspond to specific column fractions.

CTD with a high stoichiometry *in vivo* (Baskaran. 1993) and above results showed that c-Abl kinase could multifully phosphorylate the CTD *in vitro*. Also all the tyrosine residues in CTD of various RNAP II are conserved. Although serine or threonine phosphorylation of CTD has been well known and several kinases were identified as CTD phosphorylating enzymes. tyrosine phosphorylation of the CTD by c-Abl kinase and possibly other nuclear tyrosine kinases may be an important mechanism for regulating CTD function in mammalian cells.

### Interaction of RNA IIA and IIOy with the Ad-2 MLP in the minimal and complete transcriptions

In order to analyze the recruiting preference of RNAP IIA and IIOy in preinitiation complex assembly. preinitiation complex formations in the chemically defined minimal transcription system and undefined completed transcription system were carried out. Equal amounts of RNAP IIA and IIOy were incubated with the minimal general transcription factors and Ad-2 MLP and fractionated by chromatography on Sepharose CL-4B (Fig. 1). Transcription activities were also observed in excluded fractions of the column chromatography (data not shown). Figure 5 shows that RNAP IIA and IIOy are equally recruited in the preinitiation complexes (excluded fractions. lane 2~4). About 20 and 21% of RNAP IIA and IIOy are assembled in preinitiation complex (upper panel of Fig. 5). To count the nonspecifically assembled complex. complex formation experiments were performed in the absence of the Ad-2 MLP. the three general transcription factors. or both. In nonspecific conditions about 1~12% of complex was formed (1st and 2nd middle and bottom panel of Fig. 5). Also using the complete transcription system both RNAP IIA and IIOy forms are assembled in preinitiation complexes (Fig. 6. lane 3 of panel IIA and IIOy). In competition condition of RNAP IIA and IIOy. nearly equal amounts of RNAP IIA and IIOy are recruited in preinitiation complexes (Fig. 6. panel IIA/IIOy). These results showed that no discriminations between RNAP IIA and RNAP IIOy were observed in both the minimal and completed preinitiation complex assembly. It seems that the transcription on adenovirus-2 major late promoter does not distinguish between tyrosine phosphorylated and nonphosphorylated CTD. The functional significance of tyrosine phosphorylation has not been established. Tyrosine phosphorylation may play a role in the transition from complex assembly to transcript elongation. as has been proposed for serine and threonine phosphorylation. According to this model. a subset of class II promoters may recruit a tyrosine kinase. Indeed. c-Abl kinase is known to interact directly with DNA (Wang.

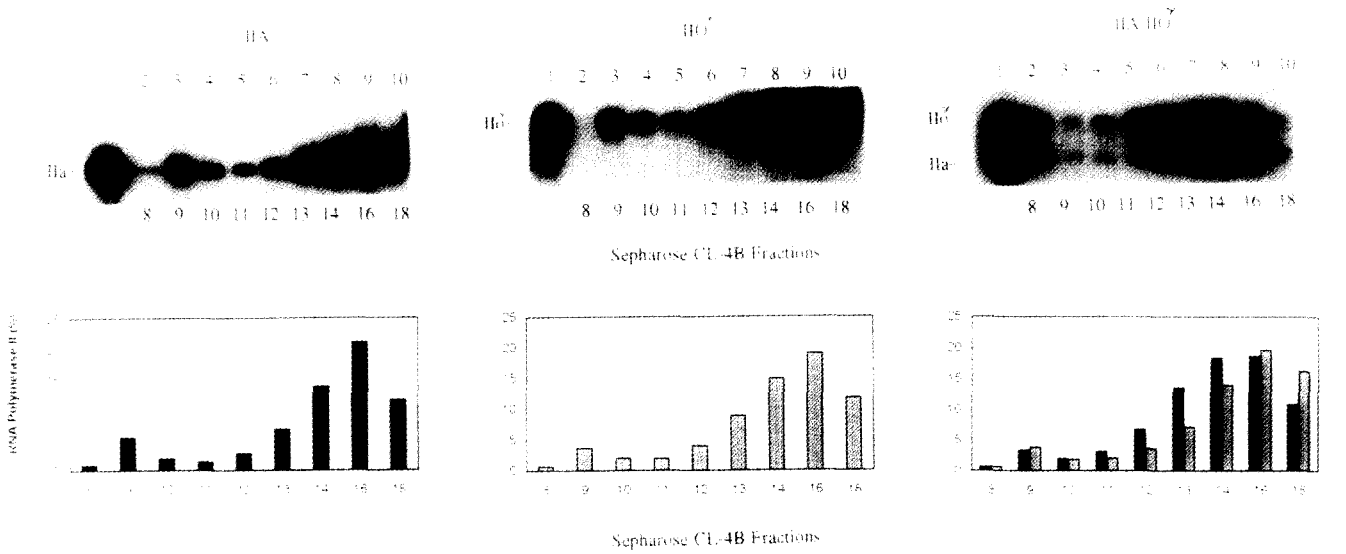


**Fig. 6.** Fractionation and quantification of preinitiation complexes formed in the presence of RNAP IIA and IIO$^{\gamma}$ (c-*abl* kinase labeled) in the complete transcription reaction conditions. Preinitiation complexes were formed and analyzed as described in Fig. 1 and Materials and Methods. The panel at the left contains aliquots of enzyme that were added to reactions in panels IIA, IIO$^{\gamma}$, IIA/IIO$^{\gamma}$. Lane 1 of each panel is an aliquots removed from the reaction at the end of the incubation. The amount of label in specific bands were quantitated on a phosphorimager. The fraction of labeled RNAP II eluting in specific fractions was calculated relative to the input (lane 1) and is presented in the bar graphs immediately below the autoradiogram. The numbers below each autoradiogram correspond to specific column fractions.

1993). It will be of interest to determine whether transcript elongation from promoters containing a binding site for c-Abl kinase is carried out by RNAP II selectively phosphorylated on tyrosine.

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