

Direct Recruitment of ERK Cascade Components to Inducible Genes Is Regulated by Heterogeneous Nuclear Ribonucleoprotein (hnRNP) K^{*[5]}

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Components of the ERK cascade are recruited to genes, but it remains unknown how they are regulated at these sites. The RNA-binding protein heterogeneous nuclear ribonucleoprotein (hnRNP) K interacts with kinases and is found along genes including the mitogen-inducible early response gene *EGR-1*. Here, we used chromatin immunoprecipitations to study co-recruitment of hnRNP K and ERK cascade activity along the *EGR-1* gene. These measurements revealed that the spatiotemporal binding patterns of ERK cascade transducers (GRB2, SOS, B-Raf, MEK, and ERK) at the *EGR-1* locus resemble both hnRNP K and RNA polymerase II (Pol II). Inhibition of *EGR-1* transcription with either serum-responsive factor knockdown or 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole altered recruitment of all of the above ERK cascade components along this locus that mirrored the changes in Pol II and hnRNP K profiles. siRNA knockdown of hnRNP K decreased the levels of active MEK and ERK at the *EGR-1*, changes associated with decreased levels of elongating pre-mRNA and less efficient splicing. The hnRNP K dependence and pattern of ERK cascade activation at the *c-MYC* locus were different from at *EGR-1*. Ribonucleoprotein immunoprecipitations revealed that hnRNP K was associated with the *EGR-1* but not *c-MYC* mRNAs. These data suggest a model where Pol II transcription-driven recruitment of hnRNP K along the *EGR-1* locus compartmentalizes activation of the ERK cascade at these genes, events that regulate synthesis of mature mRNA.

Localized spatiotemporal activation of kinases is an important mode to regulate kinase specificity (1). It has been known for more than 20 years that kinases can translocate to the nucleus (2–4), but it was not until very recently that multiple kinases were discovered to be directly recruited to genes (5–10). In fact, recent observations suggest that activation of entire canonical kinase cascades is recapitulated along gene loci (11). Compartmentalization of signaling cascades within discrete genomic regions would serve to control specificity of kinase action (12–14). In addition, direct recruitment of multiple

components of kinase cascades to a given locus may reflect local information processing microcircuits.

hnRNP K is an ancient RNA-binding protein initially identified as a component of the heterogeneous nuclear ribonucleoprotein (hnRNP)³ complex (15, 16). The K protein has been implicated to regulate many processes including transcription (17, 18), mRNA splicing (19), and stability (20). K protein in addition to RNA-binding domains contains protein-interacting modules that, on one hand, bind kinases (21–24) and, on the other hand, associate with transcription (18, 25), RNA processing (26), and translation (27, 28) factors. Moreover, the K protein-mediated interactions are regulated by signaling cascades including those initiated by mitogens (29, 30). These observations are consistent with K protein acting as a docking platform within microcircuits where it serves to integrate signaling cascades by facilitating cross-talk between kinases and factors at sites of RNA-directed processes (22, 31, 32).

The extracellular signal-regulated kinases p44 (ERK1) and p42 (ERK2) belong to the family of MAPKs (33). They are activated by a variety of mitogenic ligands that bind to surface receptor tyrosine kinases (RTKs) (13, 34). ERKs are among critical terminal effectors of kinase cascades that regulate virtually every intracellular response. Nuclear translocation of ERK1 and ERK2 is an accepted mode of their action (3–4, 35). Importantly, most of the components of the ERK1/2 signaling module have been found bound to genes in ChIP assays (11, 36, 37). ERKs target a large repertoire of nuclear protein substrates; thus compartmentalization at genomic sites explains one way by which their specificity is controlled in the intracellular environment. Still, it is not known how they are recruited and regulated along genes.

Early growth response-1 (*EGR-1*, two exons, 3,824-bp-long locus) is an immediate early gene that encodes a zinc finger transcription factor (38). *EGR-1* is rapidly and transiently expressed in many cell types following mitogenic stimulation, which then targets genes involved in signal transduction and gene expression (39). hnRNP K is recruited to inducible gene loci including *EGR-1*. Intriguingly, ERK1/2 is bound to *EGR-1* in a pattern that resembles K protein (36). Moreover, hnRNP K binds (40), regulates (41), and is a substrate (20) of ERK1/2 and

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³ The abbreviations used are: hnRNP, heterogeneous nuclear ribonucleoprotein; *EGR-1*, Early growth response-1; DRB, 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole; RIP, ribonucleoprotein immunoprecipitation; RTK, tyrosine kinase receptor; SRF, serum-responsive factor; Pol II, polymerase II; NC, noncomplementary.

TABLE 1
Antibodies list

Antibody	Catalog number	Source	Manufacturer	Peptide immunogen
Pol II CTD (4H8)	sc-47701	Mouse monoclonal	Santa Cruz	CTD
hnRNP K		Rabbit polyclonal	Ref. 32	Carboxyl terminus
GRB2	sc-255	Rabbit polyclonal	Santa Cruz	Carboxyl terminus
SOS1 + SOS2	S15530	Rabbit polyclonal	Transduction Lab	Mouse Sos1 amino acids 1–109 mouse Sos2 amino acids 1095–1297
B-Raf (C-19)	sc-166	Rabbit polyclonal	Santa Cruz	Carboxyl terminus
p-B-Raf	sc-28006r	Rabbit polyclonal	Santa Cruz	Thr(P)-598/Ser(P)-601
MEK1/2	M17030	Rabbit polyclonal	BD Transduction	
MEK1 + MEK2	ab70613	Rabbit polyclonal	Abcam	MEK Ser-218/222 and MEK2 Ser-222/226
pMEK1/2(S217/221)	9121	Rabbit polyclonal	Cell Signaling	Phospho-MEK1 (Ser(P)-217/221)
ERK1(K-23)	sc-94	Rabbit polyclonal	Santa Cruz	subdomain XI
pERK1/2	9101	Rabbit polyclonal	Cell Signaling	Thr(P)-202/Tyr(P)-204
pElk-1(S383)	9181	Rabbit polyclonal	Cell Signaling	Ser(P)-383
SRF(G-20)	sc-335	Rabbit polyclonal	Santa Cruz	Carboxyl terminus
Pol II (N20)	sc-899	Rabbit polyclonal	Santa Cruz	Amino terminus
H3	ab1791	Rabbit polyclonal	Abcam	Carboxyl terminus of human histone H3
H3K4me3	07-473	Rabbit polyclonal	Millipore	Peptide corresponding to residues surrounding and including trimethylated Lys-4 of histone H3
H3K27me3	07-449	Rabbit polyclonal	Millipore	Peptide containing the sequence AR(me3K)SAP

other components of the ERK cascade. These previously published studies may reflect compartmentalized hnRNP K-controlled bursts of ERK activity in the vicinity of inducibly transcribed genes. Here, we explored the role and interaction of hnRNP K with the ERK1/2 cascade along inducible genes following mitogenic stimulation.

EXPERIMENTAL PROCEDURES

Cells—HCT116 WT human colon carcinoma cells lines were grown in plastic six-well plates in McCoy's medium supplemented with 10% FBS (42).

ChIP Assay—Chromatin cross-linking and cell harvesting was done as described before (36, 43). Chromatin was sheared in a Bioruptor (Diagenode, Philadelphia, PA) (0.5-ml tubes) using the protocol 30-s on-off cycles for 15 min at high intensity. Chromatin immunoprecipitation assays were done using the matrix ChIP platform as described previously (36, 43).

Ribonucleoprotein Immunoprecipitation (RIP) Assays—HCT116 WT cells were grown in plastic six-well culture plates to 50–60% confluence and then rendered quiescent by lowering FBS concentration to 0.5%. The cells were treated 24 h later with warmed (37 °C) McCoy's medium supplemented with 10% FBS for the given time points. The cells were harvested, then washed once with ice-cold PBS, and stored at –80 °C. At the day of assay, the cells were lysed in 100 μ l of immunoprecipitation buffer (150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.5) containing the protease (Sigma; P5726), phosphatase (Sigma; P2850 and P8340), and ribonuclease (Sigma; R2520) (20 units/ml) inhibitors. Next, the lysates were treated with ultrasounds in Bioruptor (Diagenode) using 30-s on-off cycles for 15 min at medium intensity, and supernatants were cleared by centrifugation at 10,000 \times g/4 °C.

Ribonucleoprotein immunoprecipitation assays were performed similarly to the matrix ChIP protocol (36) with minor changes. Briefly, in-lab prepared polypropylene 96-well plates were incubated overnight at room temperature with 0.2 mg of protein A in 100 μ l of PBS/well. After a wash (200 μ l PBS/well), the well walls were blocked with 100 μ l of blocking buffer (30 min, room temperature) containing ribonuclease inhibitor (5 units/ml). The wells were cleared and incubated with 0.25 μ g of hnRNP K 54 antibody diluted in 50 μ l of blocking buffer/well

(60 min, room temperature). Samples (50 μ l of lysate) were added to wells, and plates were floated in ultrasonic water bath (60 min, 4 °C) to accelerate ribonucleoprotein-antibody binding. The wells were washed three times with 100 μ l of immunoprecipitation buffer and once with 100 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.0). The wells were incubated with 50 μ l of RNA extraction solution (QuickExtract™ RNA extraction kit; Epicenter) for 3 min at room temperature, followed by DNase I treatment performed according to the RNA extraction kit protocol. RNA samples were either stored at –80 °C or were used promptly for cDNA synthesis.

Pre-mRNA Assays—The cells were transfected with either hnRNP K siRNA or noncomplementary (NC) siRNA at final concentration of 22 nM using Lipofectamine RNAiMAX. 24 h after transfection, the cells were switched to low serum medium, and 24 h after quiescence, the cells were treated with 10% FBS for 0, 5, 15, 30, 60, and 180 min. Total RNA was extracted from cells using TRIzol® Plus RNA purification kit (Invitrogen) followed by on-column PureLink™ DNase (Invitrogen) treatment. One μ g of total RNA and random hexamers were used in cDNA synthesis. Levels of specific pre-mRNA or mature mRNAs were assessed by real time PCR using primer pairs amplifying intron-exon or exon-exon junction, respectively (see Table 2).

RESULTS

Inducible Co-recruitment of hnRNP K, Pol II, and the ERK Cascade Components along the Human EGR-1 Locus—We have previously found that mitogen-activated expression of the EGR-1 gene is associated with recruitment of ERK1/2 to this locus along with its nuclear target hnRNP K (36, 41, 44). We used a panel of antibodies to hnRNP K, Pol II, and the components of the ERK cascade (1) in matrix ChIP assays (36) to examine their spatiotemporal binding profiles along the *EGR-1* gene in the serum-treated HCT116 WT human colon carcinoma cell line (42). The results of each series of ChIP measurements are depicted in a graph grid format where each graph shows the density of an epitope (y -axis, fraction of input chromatin DNA) as a function of time following serum addition (x -axis, min).

In response to serum treatment, the Pol II level transiently increased along *EGR-1* with a peak at 15 min and returning to

TABLE 2
PCR primer list

Coordinates of PCR products refer to the NCBI36/hg18 human genome sequence.

Human <i>EGR-1</i> , -10 kb chr5:137,818,912–137,819,274	Forward Reverse	GCCACATTCAGTTCTCGCTTT TTCTCCTTCCCTGGCAACA
Human <i>EGR-1</i> , Promoter chr5:137,828,955–137,829,077	Forward Reverse	AATAAATCATAA GGTCTGCCATATTAGGGCTT ^a AATAAATCATAA TGGGATCTCTCGCGACTCC ^a
Human <i>EGR-1</i> , Ex1 chr5:137,829,326–137,829,446	Forward Reverse	AATAAATCATAA AGCTCTCCAGCCTGCTCGT ^a AATAAATCATAA GGTAGTTGCCATGGTGGGC ^a
Human <i>EGR-1</i> , Ex2 chr5:137,830,366–137,830,532	Forward Reverse	TGAACAACGAGAAGGTGCTG GGTCATGCTCACTAGGCCAC
Human <i>EGR-1</i> , Ex2.3 chr5:137,832,569–137,832,720	Forward Reverse	GCTGAGCTGAGCTTCGGTTC TCGCCCTACTCAGTAGGTA
Human <i>EGR-1</i> , +1 kb chr5:137,833,607–137,833,734	Forward Reverse	CAAGCCAAGAATCCTTCCAG CCGGATGGGAACCTTAGACA
Human <i>EGR-1</i> , +5.5 kb chr5:137,834,463–137,834,613	Forward Reverse	GGCCCAATTCTTTCTTGTGC TTTTGCCAGGAGGCTCACAG
Human <i>EGR-1</i> , Ex1-Ex2 cDNA	Forward Reverse	CAGCACCTTCAACCCCTCAG AGCGCCAGTATAGGTGATG
Human <i>EGR-1</i> , Ex1-Intron1 chr5:137,829,598–137,829,760	Forward Reverse	CAGCACCTTCAACCCCTCAG GAAACCCGGCTCTCATTCTA
Human <i>β-actin</i> Ex4-Ex5 cDNA	Forward Reverse	AGAGCTACGAGCTGCCTGAC AAGGTAGTTCTGTTGGATGCC
Human <i>c-MYC</i> , Ex3 chr8:128,822,473–128,822,545	Forward Reverse	CCTCACACCTTGGCTGAGT CCAAAGTCCAATTTGAGGCA
Human <i>c-MYC</i> , Ex1-Ex2 cDNA	Forward Reverse	CACCGAGTCGTAGTCGAGGT GCTGCTTAGACGCTGGATTT
Human <i>c-MYC</i> , Ex1-Intron1 chr8:128,818,006–128,818,075	Forward Reverse	TGCAGCTGCTTAGACGCTGGA AGGCAAGTGGACTTCGGTGTCT
Human <i>Nr4A3</i> , Ex8 chr9:101,668,741–101,668,834	Forward Reverse	TAAAGCAGGTGATTCCTCCC CCATTAAACAGCTGGTGGCTT
Human <i>Nr4A3</i> , Ex3-Ex4 cDNA	Forward Reverse	CATCGGTTTCGACGTCCTTT ACTACGGCGTGCGAACCT
Human <i>Nr4A3</i> , Ex3-Intron4 chr9:101,631,079–101,631,199	Forward Reverse	TGCAAGGGCTTTTCAAGGT ACAGCTCTCAAACCGGAAGA
Human <i>hnRNP K</i> , Ex17 chr9:85,773,503–85,773,590	Forward Reverse	TTCCCAACCCACCCCAAACT TGCTATGTCCAACAAGAGATGCAC
Human <i>hnRNP K</i> , Ex1-Ex3 cDNA	Forward Reverse	AATGCCAGTGTTCAGTCCC AGGCCCTCTTCCAAGGTAGG
Human <i>hnRNP K</i> , Ex1-Intron1 chr9:85,785,169–85,785,262	Forward Reverse	CGCAGCCTTTCAGGGAGCCC TGTCCTCACTTGTTCGCGGCC
Human <i>GAPDH</i> , Prom-Ex1 chr12:6,513,840–6,513,950	Forward Reverse	ACCTTGGGCTGGGACTGGCT AGAGAGCGAAGCGGGAGGCT
Human <i>GAPDH</i> , Ex2-Ex4 cDNA	Forward Reverse	GAAGATGGTGATGGGATTTT GAGGTGAAGGTCGGAGTC

^a Primers containing flaps (83).

base line by 3 h (Tables 1 and 2, Fig. 1, row 1, and supplemental Fig. S1). In agreement with other studies (45, 46), Pol II levels were highest at the transcription start site and decreased along the gene. At +1 kb downstream from the poly(A) site, Pol II

kinetics was similar to that seen at the end of the *EGR-1* gene. The Pol II signal was weak at the -10 kb intergenic site, suggestive of low or no transcription. The adapter protein GRB2 contains SH2 domains that bind to the phosphotyrosine resi-

hnRNP K and ERK Cascade at Inducible Genes

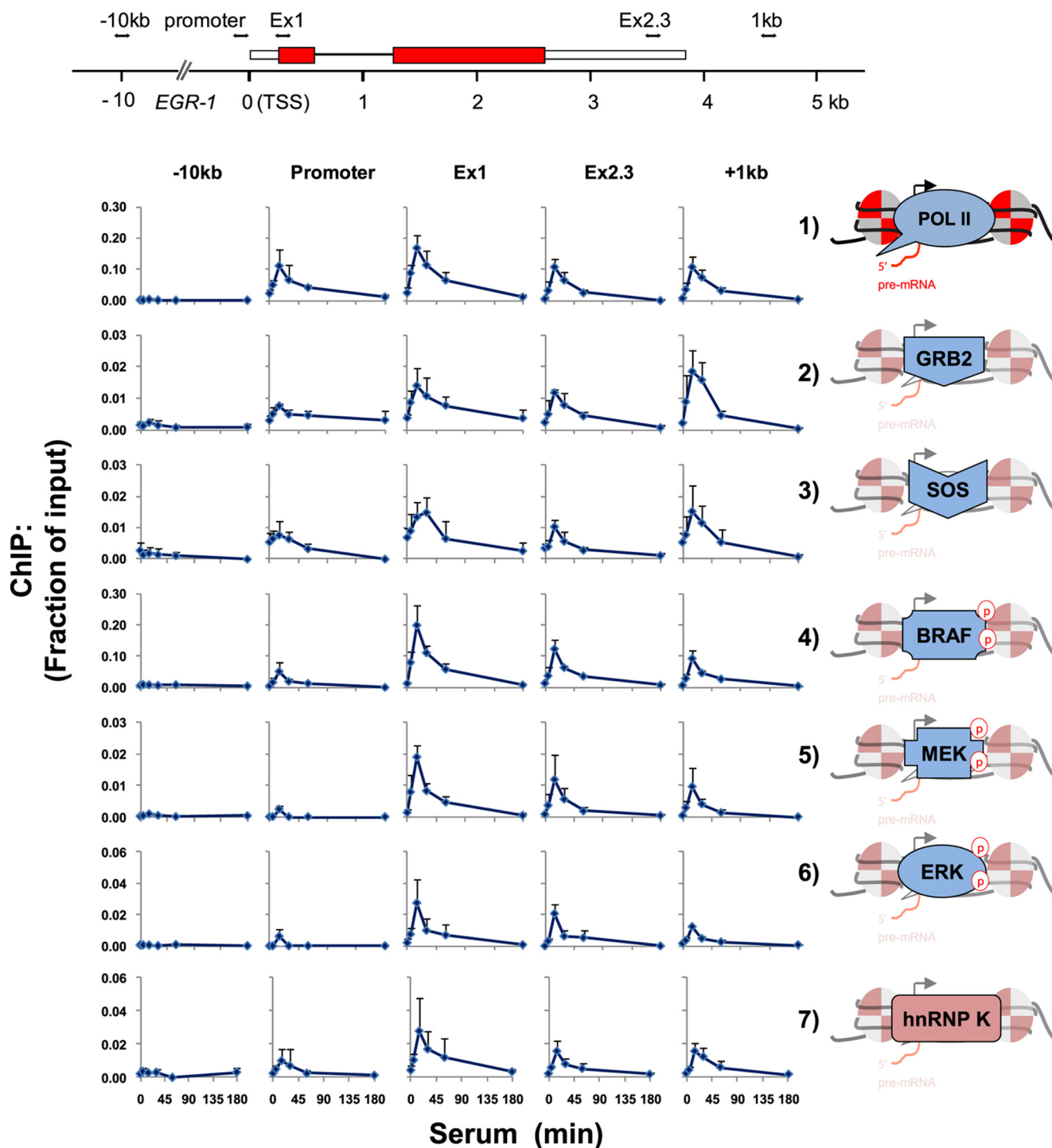


FIGURE 1. Time course binding profile of ERK cascade components, hnRNP K, and Pol II along the serum-induced *EGR-1* gene. Matrix ChIP analysis (36) of sheared chromatin from a time course of serum-treated (10% FBS for 0, 5, 15, 30, 60, and 180 min) HCT116 WT human colon carcinoma cells was done using in-lab coated protein A polypropylene 96-well plates. The antibodies used are listed in Table 1. Real time PCR was done using primers to the regions shown in the diagram of the *EGR-1* gene (the two exons are shown as boxes). The primers are listed in Table 2. The ChIP results are expressed as fractions of input DNA. The graphs represent the means \pm S.E. from six experiments. The RNA Pol II, ERK signaling components, and hnRNP K are shown to the right of the time course graphs. Protein phosphorylation is shown by a red letter *p* in a red circle. A more detailed profile is shown in [supplemental Fig. S1](#).

dues in the activated RTKs (47, 48). GRB2 also interacts directly with hnRNP K (49) and like hnRNP K is present in the nucleus. As shown in Fig. 1 (row 2) and [supplemental Fig. S1](#), GRB2 is transiently recruited all along the *EGR-1* locus, with peak levels seen at 15 min following serum stimulation, and returning to base line by 3 h. It has been shown that 0.5–1.5 kb downstream

of the poly(A) sites, Pol II pauses, allowing the recruitment of mRNA processing factors (45, 46). Interestingly, the highest inducible levels of GRB2 were present at +1 kb downstream from the *EGR-1* poly(A) signal. There was little or no binding of GRB2 at an intergenic site –10 kb upstream from the transcription start site.

Immediately downstream of GRB2 is the guanine nucleotide exchange factor SOS, which binds GRB2 via its SH3 domain. When the GRB2-SOS complex docks to phosphorylated RTKs, SOS becomes activated (47). The serum-induced pattern of SOS recruitment to *EGR-1* was remarkably similar to that observed for GRB2 (Fig. 1, rows 2 and 3, and supplemental Fig. S1). This suggests that GRB2 and SOS are recruited to the gene as a complex. Activated SOS promotes the removal of GDP from Ras, which then binds GTP and becomes active. Induced Ras activates the Raf family of protein kinases. Raf-1 (C-Raf) can form heterodimers with B-Raf, a key process that regulates their activity and involves a number of other proteins (50–52). There are reports that Ras is present in the nucleus (53). However, using different antibodies in matrix ChIP assays, we were not able to detect Ras along the *EGR-1* locus. Raf-1 is found in the nucleus (54). Activation of B-Raf kinase requires phosphorylation of the Thr-598/Ser-601 residues (52, 55). A screen using a panel of anti-Raf antibodies detected total and phosphorylated (Thr(P)-598/Ser(P)-601) B-Raf (Fig. 1, row 4, and supplemental Fig. S1, rows 4 and 5) but not the Raf-1 enzyme along the *EGR-1* gene. There was a serum-induced binding profile of B-Raf along the *EGR-1* gene, and the profile of the total B-Raf along the *EGR-1* gene and at +1 kb closely resembled that of GRB2-SOS (supplemental Fig. S1, rows 2 and 3). There was little or no binding of B-Raf at the –10 kb upstream intergenic site. Raf kinases phosphorylate and activate MEK1/2 (33). Mitogenic stimulation can induce translocation of MEK1/2 to the nucleus (4, 56). Serum treatment caused recruitment of MEK1/2 along the *EGR-1* gene and at the +1 kb downstream site; both total and phosphorylated forms were detected (Fig. 1, row 5, and supplemental Fig. S1, rows 6 and 7). No MEK was detected at the –10-kb intergenic site. MEK phosphorylates and activates ERK1/2 (4, 50). There was an inducible binding of ERK along the *EGR-1* gene and at +1 kb but not at the –10 kb intergenic site (Fig. 1, row 6, and supplemental Fig. S1, rows 8 and 9). The transcription factor Elk is known to regulate *EGR-1* expression (57, 58). ERK phosphorylates Elk at serine 383, pElk (59). We found pElk at the *EGR-1* promoter and along the gene, but surprisingly much higher levels of pElk were present at the +1 kb downstream site (supplemental Fig. S1, row 11). In contrast to the induced changes at the *ERG-1* locus, the levels of ERK components at the highly expressed GAPDH housekeeping gene were constitutively high, matched levels of mRNA and Pol II, and were not induced by serum (supplemental Fig. S2). This observation is similar to measurements made in livers (11). These results suggest that at least a pool of the active ERK components is preassembled in the nucleus. In sum, in agreement with a previous study in insulin-treated hepatocytes (11), the above ChIP assays revealed that nearly the entire activated ERK signaling apparatus (GRB2, SOS, B-Raf, MEK, and ERK) and its target transcription factor Elk is found along the *EGR-1* locus in serum-treated colon carcinoma cells (Fig. 1 and supplemental Fig. S1). Also, we obtained similar results with EGF-treated cells, suggesting that the direct recruitment of ERK cascade components to the *EGR-1* gene (data not shown) is a common feature for RTK induction of this gene.

The recruitment of the ERK cascade components along the transcribed *EGR-1* locus resembled the profiles of Pol II and

hnRNP K (Fig. 1, rows 1 and 7, and supplemental Fig. S1, rows 1 and 10). This similarity suggests that the interaction of the ERK signaling module with the *EGR-1* locus depends on transcription of this gene. This possibility was tested next.

Transcription-dependent Recruitment of the ERK Cascade Components along the EGR-1 Locus—Serum-responsive factor (SRF) initiates *EGR-1* transcription through its binding to several sites within the *EGR-1* promoter (60). We used siRNA SRF knockdown to test the effects of blocking Pol II initiation on the recruitment of the ERK cascade components to the *EGR-1* locus. In cells transfected with NC siRNA, there was constitutive and inducible recruitment of SRF to the *EGR-1* promoter, and little or no SRF binding was detected along the transcribed and intergenic regions (Fig. 2, row 1, and supplemental Fig. S3, row 1). In sharp contrast, the use of siRNA specific to SRF decreased constitutive and inducible SRF levels at the promoter. As expected, the SRF knockdown decreased serum-inducible Pol II recruitment along the *EGR-1* gene. In siRNA NC and SRF-transfected cells, the kinetics of hnRNP K recruitment paralleled the Pol II profile (Fig. 2, rows 2 and 3, and supplemental Fig. S3, rows 2 and 3), suggesting that inducible hnRNP K binding along the *EGR-1* locus is dependent on *EGR-1* transcription and thus may involve binding to the nascent transcript (61).

SRF knockdown inhibited activation and recruitment of B-Raf, MEK1/2, and ERK1 all along the gene and at the +1 kb site (Fig. 2 and supplemental Fig. S3) as well as recruitment of GRB2 and SOS1/2 (supplemental Fig. S3, rows 4 and 5). Taken together, the SRF knockdown experiments suggest that the recruitment of ERK cascade components and hnRNP K to the *EGR-1* locus is driven by transcribing Pol II.

The adenosine analog 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) inhibits the Pol II Ser-2 carboxyl-terminal domain kinase, CDK9, a component of the positive transcription elongation factor (P-TEF-b) (62–64). We used DRB to explore the role of Pol II elongation on the recruitment of ERK cascade components to the *EGR-1* gene (Fig. 3 and supplemental Fig. S4). At exon 1 (~200 bp downstream of transcription start site), DRB increased Pol II constitutive levels and spread out the duration of the inducible peak. For example, without DRB the Pol II peak was detected 15 min after serum treatment and was back to base line at 60 min, whereas with DRB pretreatment, the Pol II levels at 60 min after serum treatment were nearly as high as the levels seen 15–30 min after serum treatment. Further downstream along the gene, the DRB pretreatment decreased the amplitude and flattened the serum-induced Pol II signal, and the effect was most pronounced at the site +1 kb 3' to the poly(A) signal (Fig. 3, row 1, and supplemental Fig. S4, row 1).

The DRB effects on all of the ERK cascade components and hnRNP K mirrored the altered Pol II elongation profile along the *EGR-1* gene and at +1 kb downstream of poly(A) (supplemental Fig. S4). Thus, the DRB experiment provides further evidence that recruitment of hnRNP K and the ERK cascade transducers to the *EGR-1* locus is linked to Pol II transcription. hnRNP K regulates the ERK pathway component activity (29, 40) and could be involved in the recruitment/activation of this cascade along the *EGR-1* locus. This possibility was tested next.

hnRNP K and ERK Cascade at Inducible Genes

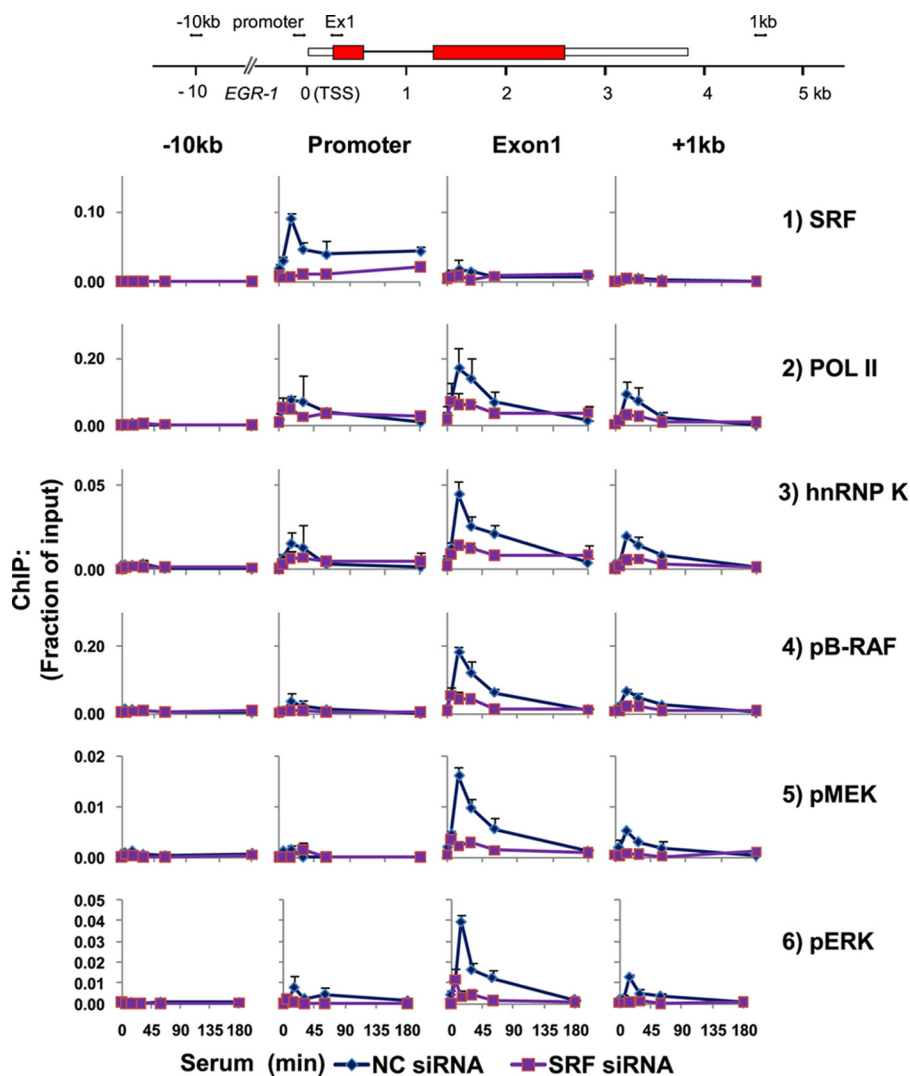


FIGURE 2. **siRNA SRF knockdown inhibits serum-induced co-recruitment of Pol II, hnRNP K, and ERK signaling cassette along the *EGR-1* locus.** The cells were transfected using Lipofectamine RNAiMAX with either SRF siRNA or NC siRNA. 24 h after transfection, the cells were switched to low serum medium, and 24 h after quiescence, the cells were treated with 10% FBS for 0, 5, 15, 30, 60, and 180 min. Matrix ChIP data are presented as fractions of input DNA (means \pm S.E., $n = 3$ experiments). A more detailed profile is shown in [supplemental Fig. S3](#).

siRNA hnRNP K Knockdown Inhibits the Serum-induced MEK Recruitment and ERK Activation along the *EGR-1* Locus—

Transfection of cells with hnRNP K siRNA decreased the total levels of hnRNP K protein in cell lysates (Fig. 4B) as well as decreased the constitutive and inducible level of K protein along the *EGR-1* locus (Fig. 4A). The siRNA K protein knockdown inhibited inducible levels of both the total and the active Ser(P)-217/Ser(P)-221 form of MEK1/2 at the *EGR-1* gene (Fig. 4A). This suggests that the MEK1/2 recruitment to the *EGR-1* gene is K protein-dependent. K protein siRNA had little or no effect on the kinetics of the total ERK1 recruitment but inhibited the inducible Thr(P)-202/Tyr(P)-204 pERK1/2 signal. This suggests that hnRNP K regulates ERK1 activity but not its recruitment along the *EGR-1* locus. Because Pol II density is also not altered at the *EGR-1* gene by siRNA hnRNP K knockdown, the inducibly bound ERK could directly interact with the Pol II (e.g. carboxyl-terminal domain). The dissociation of MEK1/2 binding from ERK1 recruitment uncovered by the hnRNP K knockdown experiment suggests that the two

enzymes are recruited to the *EGR-1* locus independent of each other.

According to the traditional view, ERK1 and ERK2 are activated in the cytosol and then move to sites of their action, including the nucleus. hnRNP K modulates MEK/ERK activity (41) and is abundant in the cytoplasm where it could regulate MEK activation of ERK prior to their nuclear translocation. To explore this possibility, we compared time course measurement of MEK and ERK phosphorylation in cell lysates (Western blots; Fig. 4B) with the pattern of their binding along the *EGR-1* gene (ChIP assays; Fig. 4A). Compared with their inducible levels along the *EGR-1* gene, the serum induction of pMEK1/2 and pERK1/2 assayed in cell lysates had low amplitude, was slower, and lasted longer. hnRNP K knockdown had no effect on the total cell MEK1/2 and ERK1 protein levels. The comparative analysis between kinase ChIP assays and Western blots revealed significant profile differences, suggesting local control of ERK by MEK activities along the *EGR-1* locus. In fact, the time course of kinase binding along the *EGR-1* looked much more

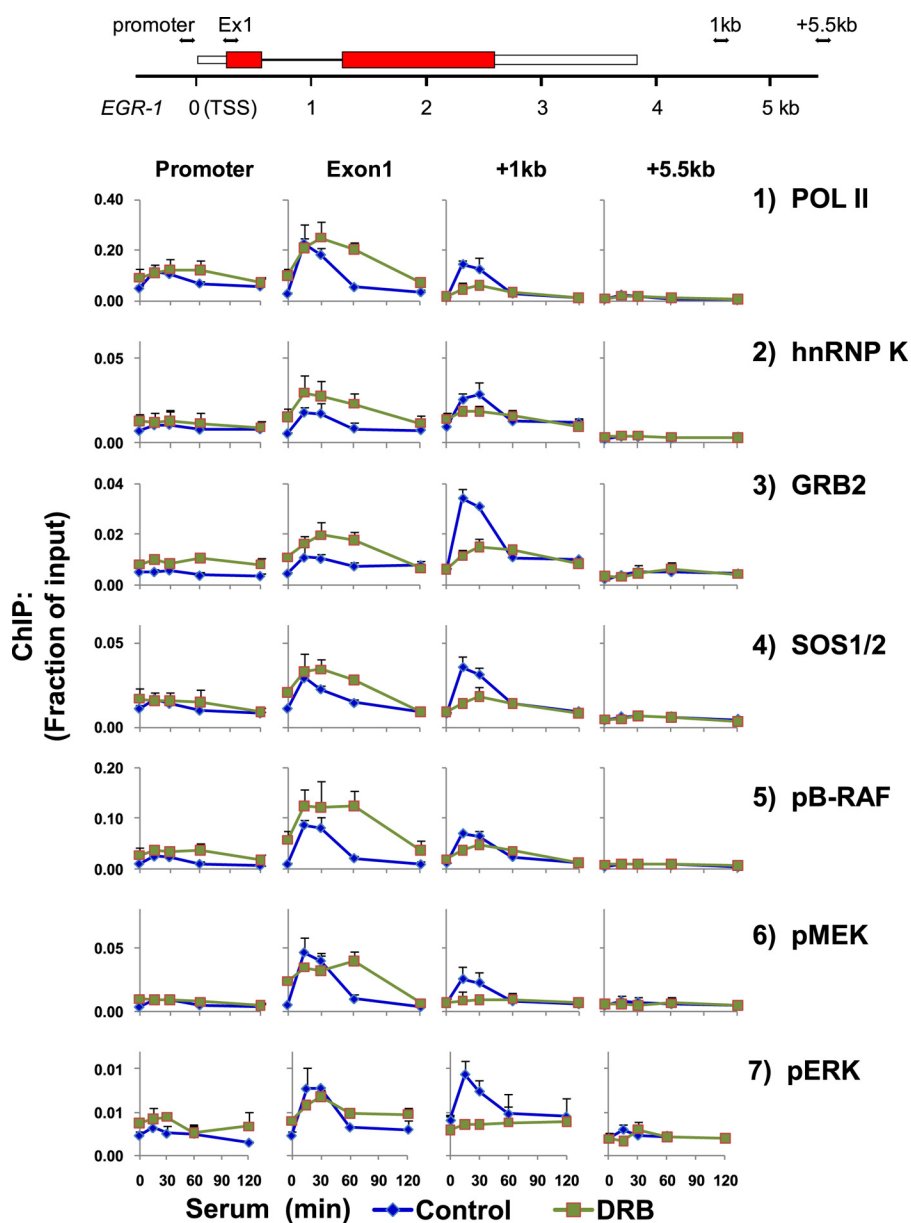


FIGURE 3. DRB-inhibited Pol II elongation decreases serum-induced levels of hnRNP K and ERK signaling cassette along *EGR-1* regions distal to the transcription start site. HCT116 WT cells grown to 40–60% confluence were made quiescent overnight by lowering FBS concentration in the medium from 10 to 0.5%. After 24 h, the cells were pretreated with DRB (50 μ M dissolved in dimethyl sulfoxide) in 0.5% FBS medium for 6 h. Dimethyl sulfoxide alone was used as control. The cells were switched to 10% FBS medium for 0, 15, 30, 60, and 120 min, at which times they were cross-linked, and chromatin was sheared. Matrix ChIP data are presented as fraction of input DNA (means \pm S.E., $n = 3$ experiments). A more detailed profile is shown in [supplemental Fig. S4](#).

like Pol II density profile (Figs. 1–3) than the Western blot pattern (Fig. 4B), suggesting that *in situ* control is linked to elongating Pol II. hnRNP K is known to interact with ERK (20) and is co-recruited with ERK cascade to the *EGR-1* gene (Figs. 1–4), and its siRNA knockdown decreases recruitment of MEK1/2 (Fig. 4A). These observations suggest that the *in situ* activation of ERK involves hnRNP K. The model of *in situ* regulation is consistent with the observation that the spatiotemporal profiles of active B-Raf, MEK1/2, and ERK1/2 at two other immediate early genes, *NR4A3* (eight exons, 45,037-bp-long gene) (65) and *c-MYC* (three exons, 5,364-bp-long gene) (66), are different from that at *EGR-1* but nonetheless also resemble Pol II density (Fig. 5, row 3) and mRNA (Fig. 5, row 1) profiles of these two serum-induced genes. Remarkably,

none of these profiles resembled the kinetics seen in whole cell lysates (Fig. 4). The local control may represent recruitment of already active components to the gene or *de novo in situ* activation. Compared with *EGR-1* activation, the profile was more strikingly different at the *c-MYC* locus where the highest levels of Pol II and kinases were observed at 3 h following serum stimulation, at a time point when the levels at *EGR-1* return to base line (Fig. 5). Moreover, although at the *EGR-1* and *NR4A3* (nuclear receptor subfamily 4, group A, member 3) genes the profiles of kinase recruitment were similar to K protein, at the *c-MYC* gene there was serum-induced kinase recruitment with little or no change in the levels of K protein. This observation suggests that at the *c-MYC* gene ERK cascade and hnRNP K protein are

hnRNP K and ERK Cascade at Inducible Genes

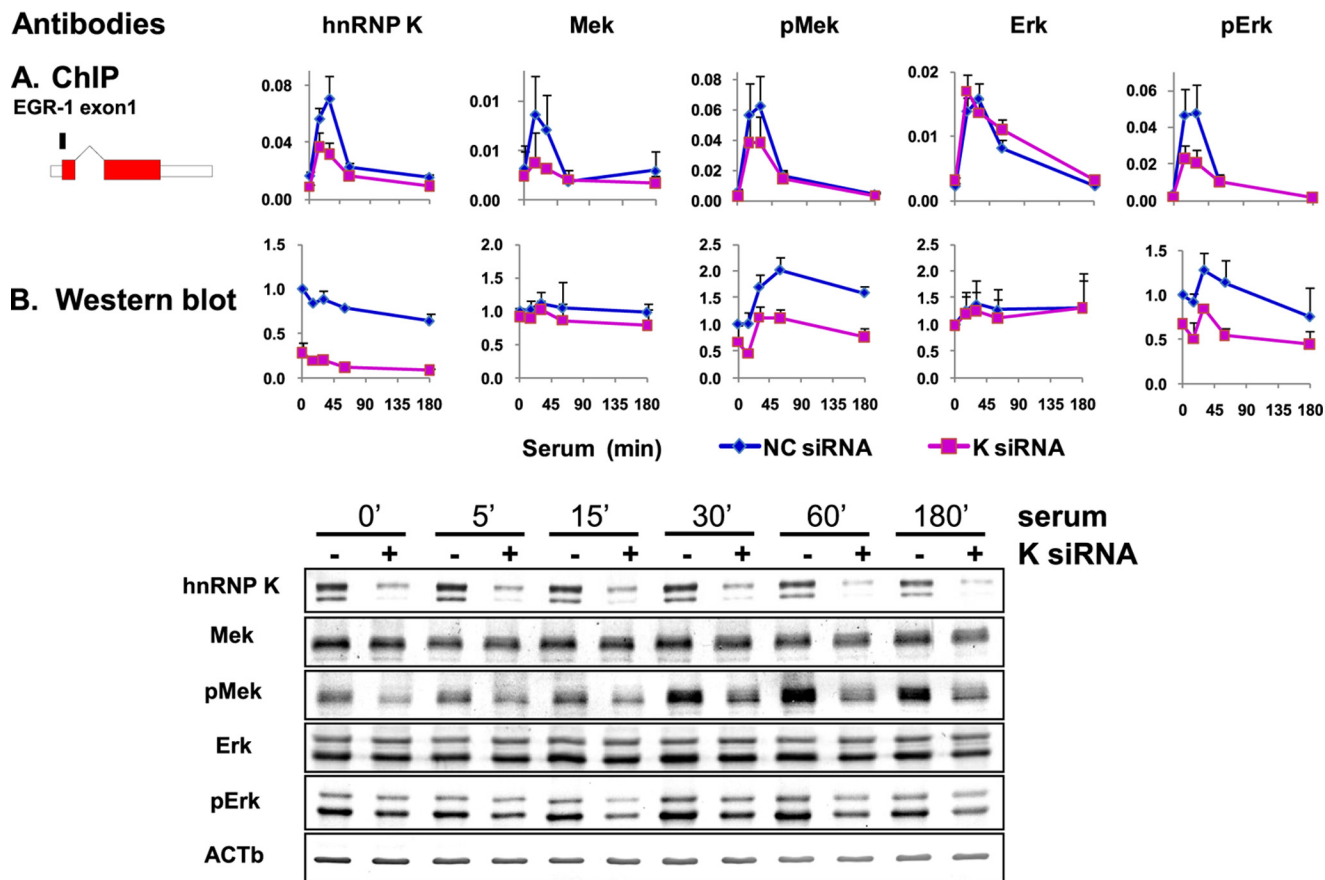


FIGURE 4. The patterns of MEK1/2 and ERK1/2 phosphorylation and the effects of hnRNP K at the *EGR-1* gene (A, ChIP) compared with whole cells (B, Western blots) are different. The cells were transfected with either hnRNP K siRNA or NC siRNA in the presence of Lipofectamine RNAiMAX. 24 h after transfection, the cells were switched to low serum medium, and 24 h after quiescence, the cells were treated with 10% FBS for 0, 5, 15, 30, 60, and 180 min. A, matrix ChIP assay was done using antibodies to either hnRNP K, total MEK1/2 (*Mek*), phospho-MEK1/2 (*pMek*), total ERK1 (*Erk*), or phospho-ERK1/2 (*pErk*). ChIP results are shown for exon 1 of the *EGR-1* gene (means \pm S.E., $n = 3$ experiments). B, cells were harvested, and lysates were resolved by SDS-PAGE and electrotransferred to PVDF membrane. Blotted proteins were assessed by Western blot analysis using the same antibodies as in A. Protein band intensities were assessed by densitometry using OptiQuant image analysis software (Packard). The data were normalized for β -actin input and expressed as fold change of 0' time point. The data represent the means \pm S.E. ($n = 2$ experiments).

recruited independent of each other and unlike *EGR-1* and *NR4A3* genes may not involve RNA binding (see below and Fig. 7).

The siRNA hnRNP K Knockdown Inhibits Serum-induced *EGR-1* Expression—We also used the siRNA knockdown to explore the role of K protein in the serum-induced *EGR-1* expression (Fig. 5). In cells transfected with hnRNP K siRNA, the constitutive and inducible *EGR-1* mRNA levels were lower compared with cells transfected with NC siRNA (Fig. 5, row 1). Interestingly, siRNA K protein did not alter the Pol II occupancy at the *EGR-1* gene (Fig. 5, row 3). siRNA K protein also inhibited serum-induced *NR4A3* mRNA expression with little or no change in Pol II density at this locus. siRNA K protein knockdown decreased the level of serum-induced ERK kinase cascade at the *NR4A3* gene. In sharp contrast to *EGR-1*, siRNA K protein knockdown appears to enhance serum-induced *c-MYC* mRNA as well as Pol II and active ERK1/2 levels without clear changes in hnRNP K density at this locus. hnRNP K knockdown also increased Pol II levels at its own locus (17 exons, 12,571-bp-long gene) without altering kinase levels. It should be noted that although siRNA markedly decreased levels of hnRNP K in cell lysates as a whole (Fig. 4) and the serum-inducible K protein at the *EGR-1* and *NR4A3* genes (Fig. 5), the

reduction K protein levels at the *c-MYC* and *hnRNP K* genes were smaller (Fig. 5, row 2). This observation may reflect the fact that a pool of hnRNP K resides within some nucleoprotein compartments (67) that are more resistant to 24–48 h of siRNA K protein knockdown. Finally, siRNA to hnRNP K decreased serum-induced pB-Raf at the *NR4A3* but not at the *EGR-1* locus (Fig. 5, row 4). Taken together, these results suggest that kinase cascade microcircuits are not the same for all inducible genes.

hnRNP K Regulates *EGR-1* Pre-mRNA Elongation and Processing—siRNA K protein knockdown inhibited serum-induced *EGR-1* mRNA expression with little or no change in Pol II occupancy at this locus (Table 3 and Figs. 5 and 6, rows 1–3). This could mean that K protein regulates expression of *EGR-1* post-transcriptionally. However, ChIP assays along transcribed regions are measures of Pol II density and not necessarily rates of elongation. Quantitative RT-PCR analysis of unspliced transcripts has been used as surrogate to estimate rates for elongation (68). We used this approach to analyze serum-induced synthesis of unspliced pre-mRNA levels as a measure of elongation rates. This experiment showed that serum-induced unspliced *EGR-1* pre-mRNA (exon 1-intron 1) was reduced by K protein knockdown (Fig. 6, row 4), suggesting that hnRNP K

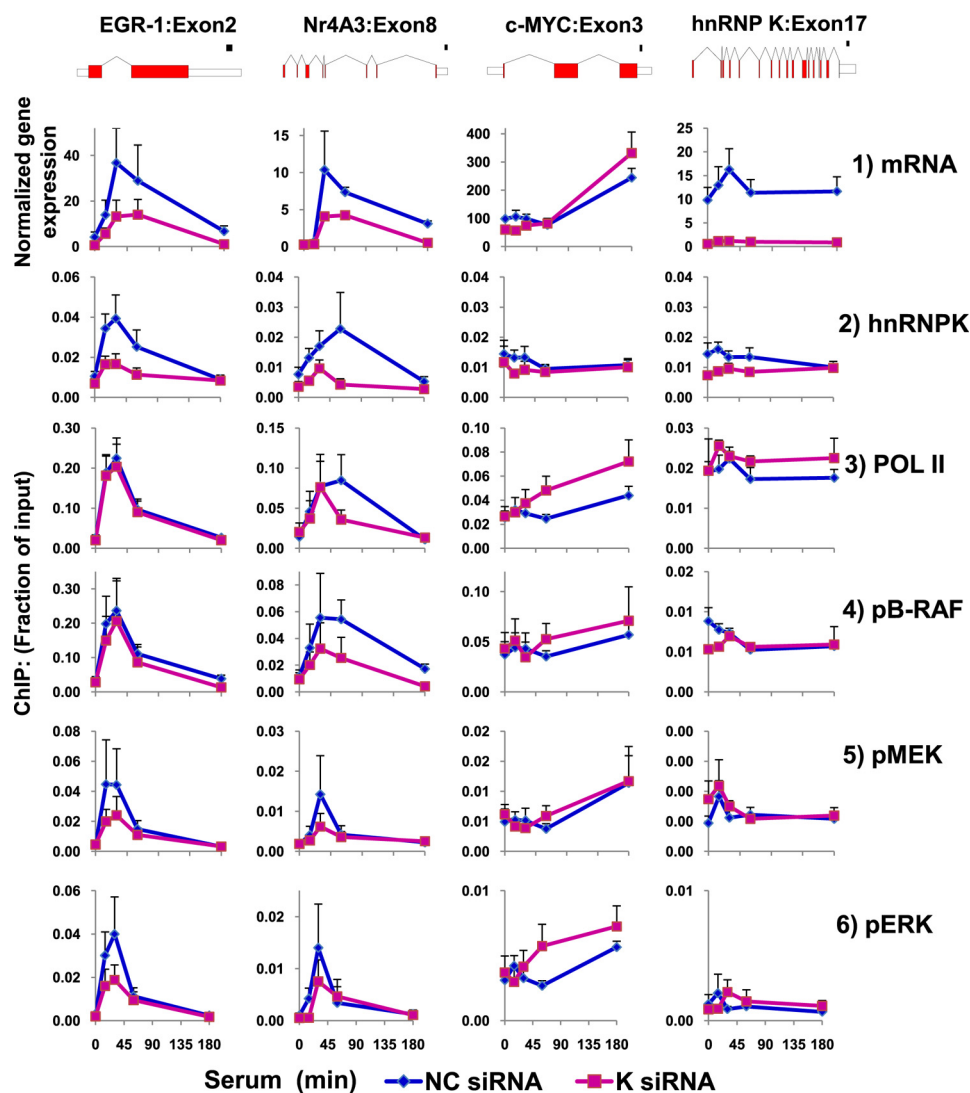


FIGURE 5. siRNA hnRNP K knockdown inhibits serum-induced mRNA levels and co-recruitment of ERK kinase cascade along the *EGR-1* and *NR4A3* but not *c-MYC* locus. The cells were transfected with either hnRNP K siRNA or NC siRNA using Lipofectamine RNAiMAX. 24 h after transfection, the cells were switches to low serum medium, and 24 h after quiescence, the cells were treated with 10% FBS for 0, 15, 30, 60, and 180 min. Total RNA was extracted from cells as described previously (85). RT reaction was carried out using random hexamers. The levels of specific mRNAs were assessed by real time RT-PCR using gene-specific primer pairs to *EGR-1*, *NR4A3*, *c-MYC*, and *hnRNP K* genes mRNAs (Table 2; means \pm S.E., $n = 3$ experiments). Chromatin was extracted and sheared. Matrix ChIP data are presented as fractions of input DNA (means \pm S.E., $n = 3$ experiments).

TABLE 3
siRNA sequences

siRNA	Sequence
SRF1	5'-GCCAGUGAGACAGGCCAUGUGUAUA-3' 5'-UAUACACAUGGCCUGUCACUGGC-3'
SRF2	5'-CCAGAGAAUGAGUGCCACUGCCUUU-3' 5'-AAAGCCAGUGGCACUCAUUCUCUGG-3'
hnRNP K	5'-AAUAUUUAGGCUCUCGGUACA*UU*-3' 5'-UGUACGGAGAGCCUUAAUAUU*UU*-3'

stimulates *EGR-1* transcription elongation. Whereas the effects of K protein knockdown along the *Nr4A3* pre-mRNA (exon3-intron4) were less pronounced, at the *c-MYC* (Myc proto-oncogene protein) locus, the levels of pre-mRNA (exon 1-intron 1) fragments were increased. Finally, K protein knockdown had no effect on its own pre-mRNA levels.

All of the steps of RNA processing appear to be linked to Pol II elongation (68–70). Both hnRNP K (19, 61) and RAS-RAF-MEK-ERK signaling cascade (71) have been shown to

regulate pre-mRNA splicing. We calculated the ratio of unspliced to spliced RNA as a measure of splicing efficiency. In cells with K protein knockdown, the ratio was higher for the *EGR-1* and *Nr4A3* pre-mRNA but not for *c-MYC* pre-mRNA (Fig. 6, row 5). These experiments suggest that hnRNP K protein regulates *EGR-1* gene transcription elongation as well as pre-mRNA splicing. These studies also underscore the heterogeneity of K protein effects on different genes.

hnRNP K Is Associated with EGR-1 and NR4A3 but Not c-MYC and hnRNP K Transcripts—hnRNP K binds RNA directly and is associated with many transcripts *in vivo* (72). We adapted the microplate matrix ChIP platform for RIP assays (“Experimental Procedures”) and used it to test whether hnRNP K binds to the *EGR-1* and *NR4A3* mRNAs in the whole cell lysates. The RIP assays showed that hnRNP K associates with the *EGR-1* and *NR4A3* but not *c-MYC* and *hnRNP K* mRNAs (Fig. 7). Taken together, these results suggest that

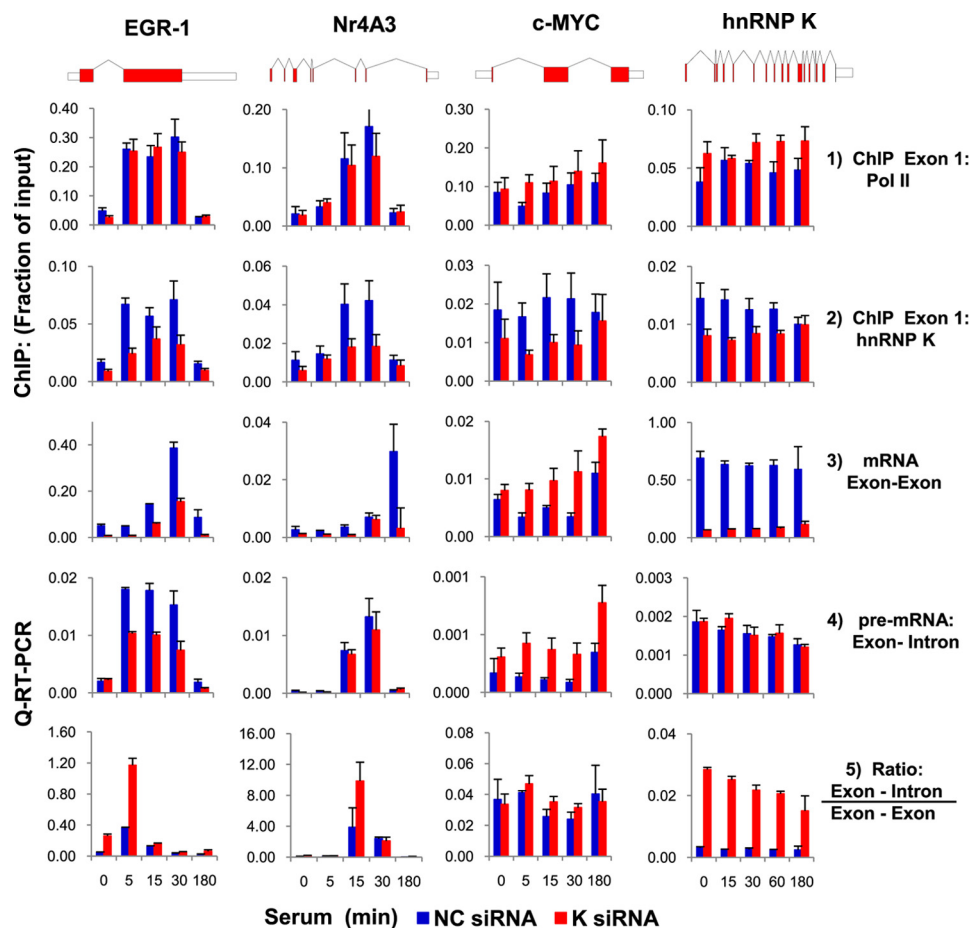


FIGURE 6. siRNA hnRNP K decreases elongation and splicing rates of *EGR-1* and *NR4A3* but *c-MYC* pre-mRNA. The cells were transfected with either hnRNP K siRNA or NC siRNA (see “Experimental Procedures” and Table 3) made quiescent and treated with 10% FBS for 0, 5, 15, 30, 60, and 180 min. Total RNA was extracted, DNase-treated, and used in RT reaction. The levels of specific mature mRNAs and pre-mRNA were assessed by quantitative (Q) RT-PCR using primer pairs amplifying exon-exon and exon-intron junction, respectively, of *EGR-1*, *NR4A3*, *c-MYC*, and *hnRNP K* genes (Table 2). The results were normalized for β -actin mRNA (means \pm S.E., $n = 3$ experiments).

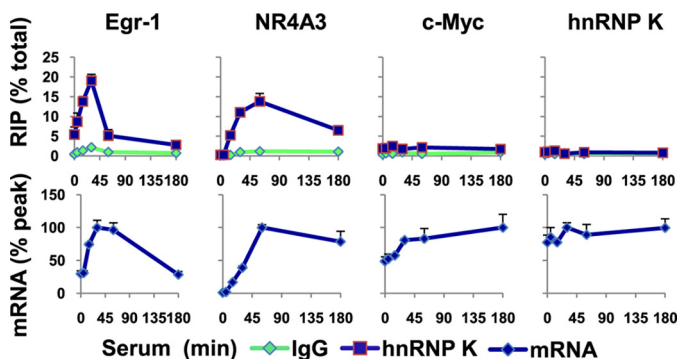


FIGURE 7. hnRNP K binds *EGR-1* and *Nr4A3* but neither *c-MYC* nor its own mRNA. *Top row*, cell lysates from a time course of serum-treated (10% FBS for 0, 5, 15, 30, 60, and 180 min) HCT116 WT human colon carcinoma cells were prepared, and microplate-based RIP was done using antibody to hnRNP K protein (see “Experimental Procedures”). RIP levels were assessed by real time RT-PCR using gene-specific primer pairs to *EGR-1*, *NR4A3*, *c-MYC*, and *hnRNP K* cDNAs. The data are expressed as percentages of average total mRNA levels (means \pm S.E., $n = 3$ experiments). *Bottom row*, total RNA was extracted from cells as described previously (85). RT reaction was carried out using random hexamers. The levels of specific mRNAs were assessed by quantitative RT-PCR using gene-specific primer pairs to *EGR-1*, *NR4A3*, *c-MYC*, and *hnRNP K* genes mRNAs (Table 2). The results are expressed as percentages of peak levels (means \pm S.E., $n = 3$ experiments).

hnRNP K regulates *EGR-1* and *NR4A3* mRNA levels co-transcriptionally through direct binding to this transcript. Thus, RNA binding may allow hnRNP K to bridge ERK cascade to the

EGR-1 and *NR4A3* genes through K recruitment to the elongating pre-mRNAs.

DISCUSSION

The discovery that terminal kinases such as ERK have hundreds of protein substrates has fueled great interest to understand mechanisms that control their specificity (13, 33). Thus far, several elements have been identified that account for kinase specificity including amino acid consensus sequence, subcellular localization or compartmentalization, duration and magnitude of the signals, cross-talk with other pathways, and scaffolds that modulate their activity (1, 12, 13). Below we discuss the elements of ERK specificity revealed in the current study and suggest a model for hnRNP-K-compartmentalized *in situ* control of ERK kinase cascade at the *EGR-1* gene, which in turn regulates K protein-dependent mRNA synthesis and processing (Fig. 8).

Compartmentalization of the ERK Cascade Signaling Module at Inducible Genes—It has been previously demonstrated that nearly all of the components of the ERK pathway translocate to the nucleus (73–76). This and another recent study from our laboratory (11) provide evidence that in fact most of the ERK cascade components, from GRB2 down to ERK, are inducibly recruited along an inducible gene (Figs. 1–3). These signal

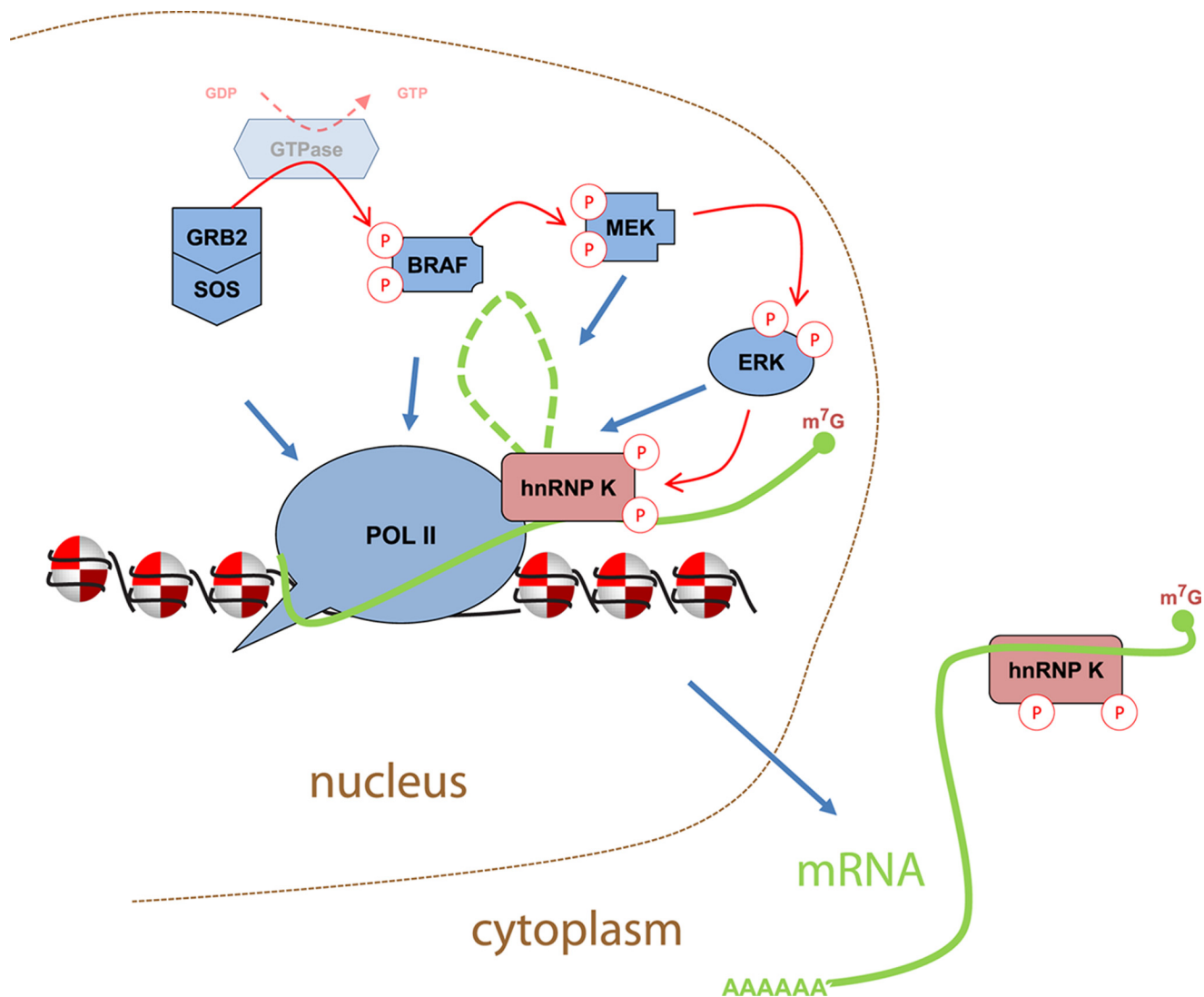


FIGURE 8. **hnRNP K-compartmentalized model of ERK activation at target loci.** Mitogen-inducible Pol II-mediated *EGR-1* transcription allows recruitment of GRB2, SOS, and active B-Raf, MEK, and ERK along the gene (Figs. 1–3). Binding/activation of MEK and MEK-catalyzed activation of ERK (Fig. 4) at *EGR-1* depends on binding of hnRNP K to elongating Pol II-pre-mRNA (Fig. 7). In turn, activated ERK phosphorylates *EGR-1* RNA-bound hnRNP K, which maintains pre-mRNA elongation, splicing, and stability and drives export of the ribonucleoprotein complex to the cytoplasm (29).

transducers were not found in the intergenic regions flanking the *EGR-1*, indicating that the ERK activation cascade is compartmentalized to discrete sites, restricting their action to only relevant gene locus-associated substrates along the Pol II transcribed region.

Timing of the ERK Cascade Induction along Inducible Genes—Under the conditions of the present study, the serum-induced levels of active MEK/ERK assessed by Western blot analysis in whole cell lysates (Fig. 4B) were relatively low. In sharp contrast, the induced ChIP levels of all the ERK cascade components at the *EGR-1* and the *Nr4A3* loci were robust and short-lived. Moreover, the temporal profiles of ERK cascade at *EGR-1* and *Nr4A3* were very different from the one at the *c-MYC* and *hnRNP K* (Fig. 5) or the *GAPDH* genes (11). The levels of active MEK/ERK seen by Western blot analysis represent the sum of MEK/ERK activities in different subcellular compartments/sites. Thus, the local burst of ERK activities occurring at different time points after mitogenic stimulation, as illustrated by the different profiles at *EGR-1*

and *c-MYC* (Fig. 5), would not be as readily detectable in bulk cell fractions.

The short burst of localized activity (e.g. at the *EGR-1* locus) would ensure that ERK will phosphorylate only those substrates that are present at the correct time in the immediate vicinity of the compartmentalized signaling cassette. The sharp peaks of activity will also provide a means for more precise cross-talk timing with other signaling cascades, further increasing specificity (52). In the cases studied here, the activation of the ERK cascade components is linked to the elongating Pol II along inducible genes. The tight coupling to Pol II seems like an ideal way for these signal transducers to specifically regulate in a timely fashion serum-induced factors involved in co-transcriptional RNA processing (Fig. 8). In fact, a recent study suggests that synchronized activation of ERK cascade components along genes appears to be important in maintaining effective transcription (11).

Activation of ERK at Inducible Gene Loci—The unexpected kinetic differences between MEK-ERK activities measured in

hnRNP K and ERK Cascade at Inducible Genes

whole cell lysates compared with those measured at the specific genomic sites (Fig. 4) are not consistent with the traditional view of cytoplasmic ERK activation. Thus, these observations raise questions about the pathways that activate ERK at these genes in the absence of comparable changes in the cell as whole. Most of the ERK cascade components (77) as well as RTKs (78–80) have previously been found in the nucleus, and mechanisms of their nuclear translocation are beginning to be defined (77). These factors could be preassembled in the nucleus so that induction of *EGR-1* and *Nr4A3* may not require serum-induced nuclear translocation of these components. We have measured high constitutive levels of ERK components at the GAPDH gene (supplemental Fig. S2) (11). We have also found high constitutive levels of insulin receptor at insulin-responsive genes without detectable insulin (11). EGFR is also bound to genes and exhibits transcriptional activity (76). Thus, RTKs could also be constitutively preassembled in the nucleus and serve to initiate activation of the ERK cascade module at inducible gene loci. Because we did not detect RAS, Ran, known to be chromatin-associated and to interact with Shc (81), could be an alternative small GTPase that could link nuclear RTKs to Grb2/SOS. We have found that a specific MEK inhibitor, U0126, blocks inducible recruitment of all components, GRB2, SOS, B-Raf, MEK, and ERK, to the *EGR-1* gene without any effects on histone marks (supplemental Fig. S5, rows 8 and 9). This observation suggests the presence of a different upstream activation ERK cascade module that indirectly controls the ERK signaling microcircuit at the *EGR-1* gene. For example, a cytoplasmic ERK activation module could drive nuclear translocation, binding, and activation of SRF, Elk (Fig. 2 and supplemental Fig. S3), and/or other transcription factors to the *EGR-1* locus initiating Pol II recruitment/elongation. In turn, hnRNP K binding activates the ERK microcircuit, a process that may involve all of the cascade components including chromatin-bound RTKs.

hnRNP K acts as a docking platform at sites of RNA-directed processes (22, 31, 82). hnRNP K exists in complexes with ERK1/2 and regulates their activities (40, 82). We show that in response to serum treatment, hnRNP K recruitment along the *EGR-1* gene is linked to Pol II transcription (Figs. 1–4). The hnRNP K knockdown inhibited MEK1/2 and ERK1/2 activities along the *EGR-1* and *NR4A3* genes (Fig. 5). This observation is consistent with a model where K protein is a functional scaffold for the ERK cascade along these loci.

ERK Targets along Inducible Genes—There could be several targets of the K protein-mediated discrete activation and recruitment of ERK1/2 along the *EGR-1* and *NR4A3* genes. Inhibition of MEK-ERK activity by hnRNP K knockdown had little or no effect on Pol II occupancy along the *EGR-1* and *NR4A3* genes (Fig. 5), suggesting that processivity was not altered. Pol II processivity can be uncoupled from the rate of elongation (84). Moreover, pre-mRNA processing is linked to Pol II elongation. Consistent with this view, we provide evidence that K protein regulates both the rate of elongation and splicing (Fig. 6). Thus, ERK cascade targets could include elongation and/or splicing factors.

ERK1/2 phosphorylates Ser-284 and Ser-353 of hnRNP K (29). Thus, after hnRNP K-mediated ERK activation along the

EGR-1 gene, the K protein itself may become ERK substrate regulating the splicing activity of K. ERK-mediated phosphorylation also drives K protein export from the nucleus (29). ERK-mediated phosphorylation of hnRNP K increases stability of K protein-bound transcripts (20) and also drives K protein export from the nucleus (29). Thus, the ERK-catalyzed phosphorylation can also play a role in hnRNP K-bound *EGR-1* mRNA stability and transport to the cytoplasm (Fig. 8).

In sum, we obtained evidence that nearly the full complement of the ERK cascade components is recruited along the inducible genes coupled to Pol II elongation. Binding of hnRNP K that accompanies these signal transducers plays a role in MEK1/2 recruitment and ERK1/2 activation along the inducible *EGR-1* and *NR4A3* loci. This study illustrates for the first time how a spatiotemporally restricted chain of events starting with Pol II recruitment followed by K protein binding to inducible loci, presumably to nascent transcript, controls kinases, which in turn regulate rate of mRNA synthesis and processing. In this regard, compartmentalized intracellular bursts of activation of kinase signaling cascades at different time points following mitogenic activation could be a more ubiquitous phenomenon than previously recognized.

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