The Rate of c-fos Transcription in Vivo Is Continuously Regulated at the Level of Elongation by Dynamic Stimulus-coupled Recruitment of Positive Transcription Elongation Factor b

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In mammalian cells, multiple stimuli induce the expression of the immediate early gene c-fos. The specificity of c-fos transcriptional response depends on the activation of signaling protein kinases, transcription factors, and chromatin-modifying complexes but also on a regulated block to elongation in the first intron. Here we show by chromatin immunoprecipitation that finely tuned control of c-fos gene expression by distinct stimuli is associated with a dynamic regulation of transcription elongation and differential phosphorylation of the C-terminal domain of RNA polymerase II. Comparison of two stimuli of c-fos expression in the pituitary cell line GH4C1, namely the thyrotropin-releasing hormone versus depolarizing KCl, shows that both stimuli increase initiation, but only thyrotropin-releasing hormone is efficient to stimulate elongation and thus produce high transcription rates. To control elongation, the elongation factor P-TEFb is recruited to the 5′-end of the gene in a stimuli and time-dependent manner. Transition from initiation to elongation depends also on the dynamic recruitment of the initiation factors TFIIIB and TFIIIE but not TFIIID, which remains constitutively bound on the promoter. It thus appears that tight coupling of signaling input to transcriptional output rate is achieved by c-fos gene-specific mechanisms, which control post-initiation steps rather than pre-initiation complex assembly.

Stimulus–transcription coupling mediates cellular responses, which require changes in gene expression such as proliferation, differentiation, or adaptive responses (e.g. neuronal plasticity) in higher eukaryotes. The induction of immediate early gene (IEG)3 transcription via multiple signal transduction pathways (1–3) is the first step in stimulus-transcription coupling. IEGs code mainly for transcription factors, which in turn will control the expression of further genes leading to the cellular responses.

Distinct extracellular stimuli can induce in the same cell a similar panel of IEGs with the same kinetics but with clear differences in their levels of expression. Therefore, during any particular stimulation, specific signal transduction and transcription mechanisms must act in a concerted manner to obtain a distinct level of gene transcription on each specific IEG. In a recent review, Hazzaline and Mahadevan (4) propose that the rate of IEG transcription varies continuously as a function of the strength of intracellular signaling events. Such dynamic control of IEG transcription implies an equally rapid reversal in addition to the well documented rapid induction of transcription.

Transcription factors constitutively present on the promoter of IEGs may function as “rheostats,” sensing the degree of activation of several signal transduction pathways and driving continuously varying levels of gene transcription. In parallel, IEG transcription is also correlated with a dynamic regulation of phosphorylation and acetylation of histone (H3/H4) tails that modify the level of chromatin compaction within the gene (5). Thus, transcription factors and chromatin components function as sensors/integrators for signal transduction inputs.

How is this information related to the basal transcription machinery, which has to modulate RNA polymerase II (pol II) to produce a continuously varying output of mRNA? We speculated that pol II features associated with either initiation or elongation could be differentially regulated by distinct signal transduction pathways. Specifically, we looked for changes in the phosphorylation levels of the C-terminal domain (CTD) of pol II during the expression of an IEG to understand how quantitative information can be converted from input intracellular signaling events to output rates of mRNA.

During the transcription cycle, transition from initiation to processive elongation and termination is associated with changes in the phosphorylation state of the CTD (6, 7). Differ-

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3 The abbreviations used are: IEG, immediate early response gene; ChIP, chromatin immunoprecipitation; CTD, C-terminal domain; FAM, 6-carboxyfluorescein; pol II, RNA polymerase II; TRH, thyrotropin-releasing hormone; Ham’s F10 serum-free medium; Ham’s F10 serum-free medium; RT, reverse transcription; TAMRA, 6-carboxytetramethylrhodamine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PIC, pre-initiation complex; cdk, cyclin-dependent kinase; RCFRD, reconstituted c-fos rat DNA; P-TEFb, positive transcription elongation factor b; PD, promoter distal; PP, promoter proximal; TBP, tata-binding protein; TF, transcription factor; GTF, general transcription factor.
ent cyclin-dependent kinases (cdks) catalyze the differential phosphorylation of the serine residues in positions 2 and 5 in the YSPTSPS repeat of the CTD. These serine residues are phosphorylated in a dynamic fashion during the transcription of the entire gene (8–11). Ser-5 is predominantly phosphorylated in pol II transcribing the 5’ part of the gene and progressively dephosphorylated during elongation while Ser-2 phosphorylation increases. Post-translational modifications of the CTD are also crucial for the maturation of the nascent RNA to mRNA in pol II genes (6, 7): RNA capping enzyme and phospho-Ser-5 CTD are functionally associated (8, 9, 12), and so are phospho-Ser-2 CTD and 3'-end RNA-processing enzymes (13, 14). CTD phosphorylation combined with other CTD modifications such as proline cis-trans isomerization (15) generates different pol II structural conformations, with major consequences on the rate of gene transcription and mRNA synthesis (16).

An important issue addressed with the present study is to understand how these different molecular mechanisms are regulated in vivo during IEG gene transcription by pol II and whether they are correlated in a quantitative manner with stimuli-dependent synthesis of mRNA.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Stimulation**—GH4C1 pituitary cells were maintained in Ham’s F10 Gluta Max medium (Invitrogen) supplemented with 2.5% fetal bovine serum and 15% horse serum at 37 °C in a humidified atmosphere with 5% CO₂. Confluent cells were incubated with serum-free Ham’s F10 Gluta Max medium supplemented with 2.5% fetal bovine serum and 15% horse serum maintained in Ham’s F10 Gluta Max medium (Invitrogen), all other PCR reactions were carried out with 100 mM Tris, pH 8.1, 1 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and collected by centrifugation. Then the cell lysate was lysed with cell lysis buffer (10 mM Tris, pH 8.1, 1 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM ortho-vanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin), and nuclei were collected by centrifugation. The nuclei were further lysed in nuclear lysis buffer (10 mM Tris, pH 8.1, 1 mM EDTA, pH 8.0, 0.5 mM NaCl) followed by rotation at 50°C for 20 minutes with an orbital shaker. The lysates were then collected by centrifugation.

**RNA Preparation and Quantitative RT-PCR**—RNA preparation, RT-PCR, standard curves, and quantification procedures have been described previously (17, 18). mRNA levels of c-fos, GAPDH, H1, and of 1S S RNA were quantified by real-time RT-PCR (Applied Biosystems). c-fos gene expression was analyzed with 200 nM forward primer 5’-TTGACAGATACGCTC-CAAGCG-3’, 200 nM reverse primer 5’-TGCTGCTGGTCTGCA-3’, and 250 nM TaqMan probe 5’-FAM (6-carboxyfluorescein)-CCGAGCTTCTCTCGTCTCAAGTGATCTGTCT-TAMRA (6-carboxytetramethylrhodamine)-3’. GAPDH and H1 mRNA levels were quantified similarly with primers and probes described for the 3’-end (GAPDH) or the 5’-end (H1) of their coding sequences (see below). The TaqMan probe 18 S RNA Human was purchased from Applied Biosystems.

**Nascent and Free Nuclear RNA Preparation and Real-time RT-PCR Analysis**—Nascent and free nuclear RNA fractions were prepared as described by (19). c-fos nascent and free nuclear RNA at different times during the stimulation by TRH was quantified with the TaqMan probes for exons 1, 3, and 4; values were normalized to 18 S RNA in each individual fraction RT-PCR (17, 18).

**Chromatin Immunoprecipitation**—GH4C1 cells were grown on 5 x 10² cells in 175-cm² flasks. Prior to stimulation, the cells were incubated with serum-free Ham’s F10 Gluta Max medium for 24 h. The cells were stimulated by the addition directly into the 30 ml of Ham’s F10 serum-free medium of 100-fold concentrated stock solutions of TRH (100 nM final) and of KCl (20 mM final) and left for the indicated times at 37 °C in a humidified atmosphere with 5% CO₂. At each time point of the stimulation, the dishes were rapidly placed on ice, and the culture medium was replaced with 30 ml of ice-cold fixing solution containing 1% formaldehyde, 9.08 mM NaCl, 4.5 mM HEPES, pH 8.0; incubation for cross-linking continued during 1 h at 4 °C, and then 127 mM glycine was added. The cells were washed 2x with phosphate-buffered saline, scraped with a rubber policeman in phosphate-buffered saline with 1 mM phenylmethylsulfonyl fluoride (PMSF), and collected by centrifugation. Then the cell lysate was lysed with cell lysis buffer (10 mM Tris, pH 8.1, 1 mM EDTA, pH 8.0, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 1 mM ortho-vanadate, 1 μg/ml aprotinin, 1 μg/ml leupeptin), and nuclei were collected by centrifugation. The nuclei were further lysed in nuclear lysis buffer (10 mM Tris, pH 8.1, 1 mM EDTA, pH 8.0, 0.5 mM NaCl) followed by rotation at 50°C for 20 minutes with an orbital shaker. The lysates were then collected by centrifugation.
DNA databases (data not shown). Rat DNA (RCFRD) was purified from chromatin (nascent transcripts) and from the nuclear fraction. Transcripts that include c-fos exon 1, exon 3, and exon 4 sequences by various stimuli is reflected in neu-
roendocrine GH4C1 cells after 30-min stimulation by 100 nM TRH, 10 nM epidermal growth factor (EGF), 10 ng/ml tumor necrosis factor–α (TNF), or 20 mM KCl. mRNA of c-fos and histone H1 as reference were quantified by real-time RT-PCR (shown are mean values ± S.E., n = 3, normalized to GAPDH). Similar results were obtained with 18 S RNA normalization. B and C, kinetic profiles of c-fos nascent transcripts (B) and free nuclear RNA (C) after stimulation of GH4C1 cells with 100 nM TRH. RNA was purified from chromatin (nascent transcripts) and from the nuclear fraction. Transcripts that include c-fos exon 1, exon 3, and exon 4 sequences (see Fig. 2A) were quantified by real-time RT-PCR (mean ± S.D., n = 3, normalized to 18 S RNA).

**List of Primer Pairs and TaqMan Probes—GenBankTM accession number and position of the 5’ sequence is indicated for each forward (F), reverse (R), and TaqMan (T) probe oligonucleotides.** The reconstituted c-fos rat DNA (RCFRD) was designed based on several rat genomic sequences present in DNA databases (data not shown). Rat H1f0 (H1 histone family, member 0; GenBankTM accession number X72624) and GAPDH (GenBankTM accession number X00972) gene sequences were selected for the analysis their gene transcription.

In Fig. 2A, the 5’ and 3’ GAPDH primer pairs and probe locations are respectively, in the exons 1 and 8 of GAPDH, 4 kb apart.

The primer and probes are as follows. For c-fos PD: RCFRD-926F, 5’-TGGCGTGCCACCTTCAGA-3’; RCFRD-972R, 5’-TGTGTAAAGGGGAGGATTG-3’; and RCFRD-970TR, 5’-FAM-AGGGCGGAGGCTTTGGGATGT-3’. For c-fos PP: RCFRD-1239F, 5’-CTCATGACGTAAGCCATCGA-3’; RCFRD-1295R, 5’-GCAATCGGCGTTGAGTATTG-3’. For c-fos exon 1: RCFRD-1423F, 5’-CCCTCGCAGGCTTGC-3’; RCFRD-1487R, 5’-GCCCTGAGTCCCCGCTTTGA-3’. RCFRD-1441T, 5’-FAM-CAAAAACGACCAGATTAGTTCTCGGTT-TAMRA-3’. For c-fos intron 1: RCFRD-1802F, 5’-CGCGCCAGTTTACTCTGA-3’; RCFRD-1871R, 5’-AGCGAGTC-TTGTCTAGAGACTTGTT-3’. For c-fos exon 2: RCFRD-2444F, 5’-CAGCCACTCTGTCTCCTC-3’; RCFRD-2531R, 5’-CGTAAAGGGGGCGCTCAGA-3’; RCFRD-2482T, 5’-FAM-CAGGGGGCGCTACTCAGAGACT-TAMRA-3’. For c-fos exon 3: RCFRD-3046F, 5’-AAGGGAGGAAATAGATGGCCTG-3’; RCFRD-3118R, 5’-CGCTTGAGGACGATCTCGTCA-3’; RCFRD-3091TR, 5’-FAM-CTCCAGTTCCGGCACTTCTG-3’; RCFRD-3723F, 5’-CTCCCCCTGGCCACCTGCA-3’; RCFRD-3746T, 5’-FAM-CTCCAGTTCCGGCACTTCTG-3’; RCFRD-3795R, 5’-TGCGGCACGTAGGAGAAGA-3’; RCFRD-3846F, 5’-CTCTCTGCTCCTCTGTCTGTTCA-3’; X00972-35R, 5’-CTGGCACTGACACAGAAAGA-3’; X00972-35R, 5’-CTGGCACTGACACAGAAAGA-3’. For GAPDH 3’ region: X00972-667F, 5’-GGGCAGCCACAGACACATCA-3’; X00972-723R, 5’-CCGTCAGCTCCTGGAGATGAC-3’; X00972-687TR, 5’-FAM-CCCTGCTACCTCGCTCTCGGCTC-TGCA-3’. For histone H1 5’ region: X72624-87F, 5’-CGGACACCGGCGAGCGAGGATCA-3’; X72624-151R, 5’-GCGGCGGCGGTCTGC-3’; X72624-115T, 5’-FAM-CAGGGAGGACGATCTCGTCA-3’; X72624-1308F, 5’-TAGGAGGACGCTGGTCTGGTTGTC-3’; X72624-1332T: 5’-FAM-CATCGATCGGTGCTCC-3’; X72624-1380R, 5’-GAATCGTGATCGGCATACGAGCA-3’, X72624-1380R, 5’-GAATCGTGATCGGCATACGAGCA-3’. For histone H1 3’ region: X72624-1308F, 5’-TAGGAGGACGCTGGTCTGGTTGTC-3’; X72624-1332T: 5’-FAM-CATCGATCGGTGCTCC-3’; X72624-1380R, 5’-GAATCGTGATCGGCATACGAGCA-3’, X72624-1380R, 5’-GAATCGTGATCGGCATACGAGCA-3’.
RESULTS

Various Stimuli Induce c-fos Transcription to Distinct Levels—We undertook to study the expression of the c-fos oncogene, a highly inducible IEG for which transcription control at the level of initiation and a block to elongation situated in the first intron are well documented (17, 20–22). However, none of those earlier studies have addressed the dynamic control of this block with different stimuli in vivo.

The ChIP experiments shown in Figs. 2 and 3 used three different anti-pol II antibodies (8WG16, H14, and H5), which recognize different epitopes of the CTD. 8WG16, which binds non-phosphorylated CTD, was used to follow total pol II, assuming that among the 52 heptad repeats of the mammalian CTD there will always be some that are not phosphorylated. ChIP data obtained with 8WG16 and with a different anti-pol II antibody were indeed indistinguishable (data not shown). H14, recognizing phospho-Ser-5 CTD and H5, directed against phospho-Ser-2 CTD distinguished between phospho-Ser-5 CTD and doubly (Ser-2/Ser-5) phosphorylated CTD plus phospho-Ser-2 CTD. A comment on the specificities of these commonly used antibodies 8WG16, H14, and H5 may be found in the supplemental material.

The pol II distribution on exons 1, 3, or 4 at different times during the activation and attenuation phases of TRH-induced c-fos gene transcription was quantified by real-time PCR with the corresponding TaqMan primers and probes (Fig. 2A). Prior to stimulation, total pol II and phospho-Ser-5 CTD forms of pol II were present on exon 1 indicating a certain level of constitutive transcription initiation (Fig. 2, B and C). However, pol II

Dynamic Control of Elongation Determines the Rate of c-fos Transcription—Quantitative analysis of c-fos nascent transcripts showed a very rapid rise in transcription rates that reached maximal levels at 12 min of stimulation (Fig. 1B). Beyond this point, transcription was attenuated but remained significantly elevated over starting levels for up to 96 min after addition of TRH. Consequently, a peak for c-fos mature transcripts in the free nuclear RNA fraction was observed for ~24 min; thereafter, an elevated steady-state level of c-fos mRNA was maintained (Fig. 1C).

To understand how transcriptional rates are dynamically controlled we quantified at various time points the distribution of pol II on the c-fos gene and assessed the phosphorylation of its CTD tail. Combining ChIP with quantitative real-time PCR made this possible in intact cells. In particular we could specifically address whether continuous dynamic control is exerted upon the transcription elongation steps. Indeed previous work, including our own, had suggested that Ca2+ signals could relieve a block to elongation in the first intron of the c-fos gene (17, 20–22). However, none of those studies have directly addressed the dynamic control of this block with different stimuli in vivo.

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Stimulus-coupled Transcription Elongation

RNA Polymerase Transcribing Constitutively Expressed Genes Is Unaffected during Stimulation of c-fos Transcription—Changes in CTD phosphorylation upon transcription activation were observed so far for inducible genes known to harbor a promoter proximally paused pol II (10, 11). We wondered whether pol II on unregulated genes would show an altered kinetic pattern of CTD phosphorylation after stimulation by TRH. We choose two housekeeping genes known to be regulated differentially at the level of elongation: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which presents an elongation block in the 5′-end, and Histone H1 which does not (10, 26). Nascent transcript RT-PCR and ChIP assays were performed as in Figs. 1 and 2 with TaqMan probes corresponding to the 5′ and 3′ transcribed sequences of both housekeeping genes (Fig. 3A). No further induction of gene transcription was observed for GAPDH and H1 after stimulation by TRH; nascent or free nuclear transcripts of both genes were constant (Fig. 3A). TRH modified neither pol II density nor CTD phosphorylation on either gene (Fig. 3, C–E). However, a clear difference in spatial pol II distribution was observed between GAPDH and H1: for GAPDH, pol II, both total (8WG16) and phospho-Ser-5 CTD (H14), was more abundant in the 5′ region, consistent with paused polymerases and a block to elongation. This difference is not observed for phospho-Ser-2 or dually phosphorylated CTD (H5), attesting to the earlier observations (10) that such modified pol II will be competent to elongate transcription to the 3′-end of the GAPDH gene. Illustrates that the kinetics of transcriptional rates result from the dynamic control of elongation.

In summary, c-fos like many other immediate early genes displays biphasic kinetics of gene expression after extracellular stimulation. Induction of c-fos by TRH is characterized by a short and transient phase of very active gene transcription (9–12 min) followed by a longer attenuation phase. CTD phosphorylation of initiated and of elongating pol II changes during the different phases of gene transcription. At the peak of active gene transcription CTD is highly phosphorylated both on Ser-2 and Ser-5; thereafter, for pol II on exon 1, phospho-Ser-2 and doubly phosphorylated pol II (H5) decreased, whereas phospho-Ser-5 (H14) was stable (Fig. 2, C and D). Like for CTD kinases (9, 10) this suggests that the control of elongation could be also regulated by specific CTD phosphatases in a position- and time-dependent manner. Such phosphatases able to distinguish between the Ser-2 and Ser-5 positions have already been described (25).

was not detected on exons 3 and 4, consistent with a block at the level of elongation in resting cells (17, 20–23). Stimulation with TRH led to a rapid increase of total pol II distribution all along the gene until 9–12 min of stimulation (Fig. 2B). Strong transcription activation occurring during this period is correlated with a high level of Ser-5 and Ser-2 phosphorylation of the CTD of pol II (Fig. 2, C and D). Both initiation and elongation are stimulated during this phase of c-fos gene transcription. After 12 min, transcription was attenuated, and pol II density was decreased on exons 3 and 4 with a reduction in the number of pol II phosphorylated on the two serine residues. Interestingly at 48 min, the level of total pol II (8WG16) and Ser-5-phosphorylated pol II (H14) in the exon 1 was still high compared with 9–12 min of stimulation (Fig. 2, B and C). This result suggests that the recruitment and initiation of pol II at the promoter continues over a long period even though the amount of elongating pol II has decreased. Hence, following stimulation by TRH, temporal control of transcription initiation may be distinct from the control of transcription elongation.

Elongation can be best illustrated by the ratio between pol II ChIP signals from exon 3 versus exon 1 (Fig. 2E). For total pol II (8WG16), this ratio increased during the activation phase demonstrating that elongation becomes more efficient to drive pol II than new recruitment. Following the peak of transcriptional rates, the ratio decreased progressively to a new steady state, which was maintained. The ratio curve was strikingly parallel to the kinetics of transcriptional rates (Fig. 2E versus Fig. 1C). This illustrates that the kinetics of transcriptional rates result from the dynamic control of elongation.

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FIGURE 3. Histone H1 and GAPDH gene transcription rates are unaffected by TRH and result from stable transcription initiation and elongation. A, histone H1 (i.e. H1F0, family member 0) genomic locus and GAPDH transcribed sequence with the 5′ and 3′ sequences amplified for quantification by real-time RT-PCR (green versus yellow, and blue versus violet, for H1 and GAPDH, respectively). B, kinetics of nascent RNA (NR) and free nuclear RNA (FNR) of H1 and GAPDH after stimulation with TRH, determined as in Fig. 1 (B and C). C–E, kinetics of pol II density (C) and CTD phosphorylation (D, Ser-5; E, Ser-2) for promoter proximal (5′) and distal (3′) regions on histone H1 and GAPDH genes, analyzed by real-time PCR after ChIP as in Fig. 2 (B–D), expressed as percent of input (mean ± S.E., n = 3).
Taken together, the ChIP experiments with anti-pol II antibodies show clearly that the dynamic control of elongation reflected by the kinetics of pol II occupancy of coding sequence is a gene specific phenomenon. Whereas constant processes that are independent of extracellular signals are operating on the GAPDH and H1 genes, the c-fos gene must contain the information to regulate dynamically CTD phosphorylation and elongation after extracellular stimulation. This information may not be restricted to the gene promoter (27).

Distinct Stimuli Activate Differentially Initiation, Elongation, and Ser-5 versus Ser-2 CTD Phosphorylation—To confirm that different stimuli or signal transduction pathways activate distinct mechanisms of CTD phosphorylation, we performed pol II ChIP experiments on cells after depolarization with 20 mM KCl (Fig. 4). KCl increased pol II density in the 5′ region of the gene like TRH and with similar kinetics. In contrast, pol II density in the 3′ region remained very low (Fig. 4A).

Phospho-Ser-5 CTD pol II (H14) in the exon 1 increased at a similar rate between KCl and TRH (compare Figs. 4B with 2C) but remained almost absent on exons 3 and 4. Only very limited CTD Ser-2 or dual phosphorylation was seen after KCl stimulation (compare Figs. 4C with 2D). In summary, depolarization with KCl caused substantial recruitment of pol II to the 5′ region of the c-fos gene, where CTD was phosphorylated on Ser-5; however, this weak stimulus could not promote elongation or the associated dual phosphorylation of CTD on Ser-2 and Ser-5. Similar observations have been made for tumor necrosis factor (data not shown).

One of the key players in this transition to productive elongation is the positive transcription elongation factor b (P-TEFb), which is composed of cyclin T and the cyclin-dependent kinase 9 (cdk9), which phosphorylates CTD on Ser-2. We looked here for a difference in P-TEFb recruitment between TRH and KCl stimulation. ChIP experiments with an anti-cyclin T antibody (Fig. 5) showed that, prior to stimulation, cyclin T was almost absent on the c-fos gene. At the peak of TRH-stimulated transcription (12 min) P-TEFb was massively recruited to the whole transcribed sequence. After attenuation (TRH 48 min), the levels of cyclin T decreased, consistent with a reduction in the elongation efficiency. KCl caused only very limited recruitment of P-TEFb to the c-fos coding sequence. Taken together these results suggest that the control of c-fos elongation by different stimuli occurs principally in the 5′ region by post-initiation mechanisms. Stimulus-dependent recruitment of P-TEFb is thus linked to pol II occupancy of the c-fos gene and to its capacity to overcome the block to elongation in the first intron.

Activation and Attenuation of c-fos Transcription Involve Differential Pre-initiation Complex Assembly—Recruitment of pol II to the exon 1 of c-fos and pol II Ser-5 CTD phosphory-
tion are sustained during the attenuation phase (12–48 min) of TRH-stimulated gene transcription (Fig. 2). This result could be attributed to either (i) a constant level of transcription initiation by TRH or (ii) the consequence of the block to elongation situated in the first intron (17, 20–23). Indeed in the second case, it’s possible that the reduction of pol II occupancy (observed in Fig. 2D, TRH 48 min) may result in elongation complexes more susceptible to pause or arrest in the intron 1 and progressive stacking of pol II in the 5′-end of the gene, even if transcription initiation is reduced.

To determine which mechanism operates during the attenuation of c-fos transcription, we evaluated the intensity of transcription initiation by performing ChIP experiments with antibodies directed against general transcription factors. To analyze PIC assembly in detail we used four sets of primers that amplified the following sections of the 5′ regions of the c-fos gene: promoter distal (PD), promoter proximal (PP), exon 1, and intron 1, the amplicons being centered, respectively, at −350 (PD), −40 (PP), +130 (exon 1), and +550 (intron 1) (Fig. 6A) of the start site. Commercially available antibodies directed against TBP, TFIIH, TFIIE, TFIIF, and Cdk8 were used. Chromatin was prepared from cells fixed prior to or after 12 and 48 min of TRH stimulation.

The ChIP data in Fig. 6 (C–G) show the presence on the proximal promoter of all GTFs tested prior to stimulation (time 0). The ChIP signal from the proximal promoter is always stronger than from other sections and minimally five times higher than the mock signal. TBP, TFIIH, TFIIE, TFIIF, and Cdk8 are thus present in the minimal promoter, sufficient to recruit a relevant amount of pol II and initiate basal c-fos gene transcription prior to any stimulation (Fig. 6A).

Interestingly the composition of the PIC and the interactions of these different initiation factors with the c-fos gene change during TRH stimulation. Whereas TBP binding remained constant and limited to the proximal promoter (Fig. 6C) TFIIB, TFIIE, TFIIF, and Cdk8 binding to the proximal promoter was enhanced in parallel to the enhanced recruitment of pol II (Fig. 6, B, D–G). This increase is moderate for TFIIE and Cdk8 but quite marked for TFIIF and TFIIE. Thus during the maximal acceleration of c-fos transcriptional rates, there was a marked change in the composition of GTFs associated with the proximal promoter. This change was reversed during the subsequent attenuation of transcriptional rates, such that at 48 min TFIIE, TFIIF, Cdk8, and TFIIB abundances at the proximal promoter were back to those seen prior to stimulation; yet only TFIIE remained slightly more elevated.

Therefore, because different levels of PIC are assembled between 12 and 48 min of TRH stimulation, this suggests that initiation is probably not maintained to a constant level during the attenuation phase of gene transcription. c-fos transcription initiation at 48 min even seems to return to the basal state when comparing PIC assembly for both conditions. pol II present in the exon1 (Figs. 2 and 6B, TRH 48 min) are indeed pol II stacked in the 5′-end due to pause/arrest sites. Between 48 and 96 min the elongating fraction of pol II maintained nascent transcripts levels slightly elevated compared with the basal state (Fig. 2D and 1B). Thus the control of c-fos transcription rate is mainly achieved here at the level of elongation by stimulus coupled mechanisms.

Interestingly ChIP performed for TFIIE and TFIIH shows their significant association with the exon 1 and intron 1 sections during the active phase of gene transcription (Fig. 6, D and G, TRH 12 min), whereas others (TFIIB and TFIIID) are exclusively present on the proximal promoter. TFIIE, TFIIF, and TFIIH, in addition to their role in PIC formation, can cooperate to suppress promoter-proximal stalling facilitating the transition of pol II to productive elongation (28–30). Thus TFIIE/TFIIH recruitment to the block site may modulate pol II in a stimuli-dependent manner like for P-TEFb (Fig. 5).

DISCUSSION

This quantitative assessment of the dynamics of pol II positioning and CTD phosphorylation on the immediate early response gene c-fos in intact mammalian cells highlights the
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**FIGURE 7. A model for transcription regulation of \( c\text{-}fos \) in neuroendocrine cells.** For explanation refer to text under “Discussion.” Blue \( P \), phospho-Ser-5 on the CTD of pol II; red \( P \), phospho-Ser-2 on the CTD of pol II; red triangle: block to elongation in the first intron; dashed line, moderate recruitment; solid line, strong recruitment, and gray, stimulus-coupled factors.

independent regulation of elongation, which appears to be the decisive element controlling stimulated transcriptional output. This is most obvious from the comparison of two stimuli, KCl and TRH, with KCl resulting in much lower \( c\text{-}fos \) mRNA output than TRH. Although both stimuli result in recruitment to exon 1 of pol II, which is significantly phosphorylated on the Ser-5 residues of the CTD, only TRH can boost the elongation machinery. Tight coupling of signaling input to transcriptional output is thus achieved by mechanisms that control elongation rather than initiation and pre-initiation complex assembly. The latter, in part constitutive and for the rest slowly reversible, is merely a prerequisite for finely tuned elongation control to be effective. Among the mechanisms that control elongation, the selective phosphorylation by P-TEFb of Ser-2 in the heptad repeats of the CTD of pol II stands out.

Transcription initiation and elongation were distinguished based on the dynamic changes of the distribution of pol II, total, and specifically phosphorylated forms, in the coding region of the \( c\text{-}fos \) gene. Initiation is defined as the recruitment of pol II to the promoter and the synthesis of the first RNA phosphodiester bonds, which are molecular mechanisms that include CTD Ser-5 phosphorylation (31, 32). To further characterize the initiation steps necessary for \( c\text{-}fos \) transcription, we investigated the assembly of the PIC by looking for the association of specific GTFs with the 5’ region of the gene (Fig. 6). We found specific qualitative and quantitative changes in the PIC associated with the stimulation of elongation, which we describe in the scheme in Fig. 7. Three states of the system are depicted, namely a basal state, a state of fully stimulated transcriptional rate (12 min after TRH), and an intermediate state seen either 48 min after TRH (attenuated steady state) or with the weak stimulus KCl.

The basal state is characterized by a moderate but significant presence of pol II on the whole 5’ region of the \( c\text{-}fos \) gene, with CTD Ser-5 in part phosphorylated. TBP is bound to the proximal promoter. A clear association of CDK8, TFIH, and TFIIE with all sections of the 5’ region of \( c\text{-}fos \) was detected by ChIP (Fig. 6); however, the signal was weak (except TFIIE), most likely because it was based upon protein-protein rather than protein-DNA interactions. In the model, pol II initiated in the basal state will not elongate the nascent transcript beyond the first intron. Transcription will be arrested most likely in the region of the intron 1 (+363 to +387 nucleotides from the start site), which was defined as a “block to elongation” (20, 33). As a consequence basal transcription rates will be very low (<0.5% of fully stimulated rates).

The fully stimulated state is characterized by the recruitment to the proximal promoter, exon 1, and intron 1 of additional pol II with CTD Ser-5-phosphorylated. The recruitment of CDK8, TFIH, and TFIIE is reinforced in these sections. Most likely these GTFs are associated with pol II, TFIIB is present on the proximal promoter. The transient association of TFIIB and TFIIE with the constitutive TFIID and TFIIE factors may stabilize PIC assembly causing higher re-initiation rates. In addition this reconfiguration of GTFs at the promoter may favor the initiation-elongation transition by strongly recruiting P-TEFb to the same site. P-TEFb stimulates pol II by phosphorylating Ser-2 residues in the CTD. The regulatory process important for elongation is most likely occurring at the promoter, post-initiation, and probably involves tight coupling between GTFs and elongation factors. pol II strongly phosphorylated on both Ser-5 and Ser-2 of the CTD associated with P-TEFb can be demonstrated by ChIP on all exons of the \( c\text{-}fos \) gene. Such a high occupancy by pol II of the \( c\text{-}fos \) gene results in a spectacular rise in its transcriptional rate.

The intermediated stimulated (KCl) or attenuated state (TRH 48 min) depicted in Fig. 7 (bottom) was characterized by a markedly reduced TFIIB binding to the proximal promoter and an equally strikingly reduced P-TEFb association. However, pol II was strongly present on the part of the gene upstream of intron 1. The CTD of pol II was phosphorylated on Ser-5 and to a much lower extend also on Ser-2. Hence a much lower fraction of pol II initiating transcription of the \( c\text{-}fos \) gene can elongate its transcripts. As a consequence, a large number of pol II complexes remained stacked on exon 1 and intron 1. Indeed, the global analysis of pol II distribution shown in Fig. 2 showed a sustained presence of pol II on exon 1 following the attenuation of the TRH response and with KCl. Although this presence may in part be due to sustained initiation, it may be partly explained by an enhanced stacking of pol II upstream of the block to elongation. This “cocked" status of the machinery implies that, upon new signaling, input changes in transcrip-
tionary rate can immediately follow. For neuroendocrine cells and neurons, c-fos transcription is never stopped completely under physiological conditions. The transcription machinery is thus most likely in the status depicted in Fig. 7 (bottom).

As we showed in this study, kinetics of pol II occupancy of the 5’ part of the c-fos gene following TRH was strikingly parallel to those for phospho-Ser-2 CTD pol II and P-TEFb interactions with exons 3 and 4 (Figs. 2D and 4C). TRH, in contrast to KCl, could very effectively recruit the positive elongation factor P-TEFb to the coding region of the gene. We are currently investigating the signaling pathway downstream of the TRH receptor, which may explain enhanced P-TEFb recruitment. Direct activation of elongation factors by signaling protein kinases has not been found so far. However, interactions between distinct signaling pathways and the elongation machinery may be indirect. Indeed previous reports have shown that the combination of several transcription factors with coactivators/repressors and the general transcription machinery is known to regulate differently pol II elongation via the recruitment of elongation factors (34–38). Here, for c-fos, we provide evidence that some initiations factors (TFIIB and TFIIE) may also play a role in the organization of an elongation-competent pol II complex following TRH stimulation. Other factors like TFIID are more important for PIC assembly.

For a majority of genes it is of decisive importance to be “on” or “off” at the right place in the right moment. Such binary control requires sophisticated promoters but need not imply any dynamic elements. In contrast, for IEGs, continuous adjustment and precision of the rate of transcription is of paramount importance (4). As we show here, the dynamic element is contributed by the mechanisms controlling elongation. Such mechanisms have evolved to coordinate transcription with RNA maturation, two processes that are complex and highly dynamic and for which diversified phosphorylation of the CTD of pol II is of central importance (16, 39). Here we show that the input of signaling into transcriptional elongation coincides with gene-specific phosphorylation and dephosphorylation of the CTD of pol II. For c-fos, this process is of quantitative importance, because a finely tuned transcriptional rate permits extracellular stimuli to adjust the levels of c-fos mRNA. For other genes, e.g., Homer 1 and fibronectin (40, 41), cell activity and stimuli can influence splicing and change mRNA species presumably via the same mechanisms.

Substantial work still lies ahead to identify kinases and phosphatases that may couple signaling cascades to CTD phosphorylation and elongation, e.g., via the dynamic recruitment of P-TEFb with cdk9 phosphorylating the decisive Ser-2 residues. Most intriguing is the gene-specific nature of CTD phosphorylation and elongation control (cf. c-fos versus histone H1 and GADPH, Figs. 2 and 3). It appears that intragenic sequences can contribute (42), consistent with the important contribution of intragenic elements to the control of c-fos expression by intracellular signals (27). In summary, the work on transcription control of the last years highlights the complexity and versatility of the molecular interactions based on which dynamic signals from outside and inside the cells differentially control quality and quantity of transcriptional output, using information on the entire gene.

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