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Essay

Regulation of Eukaryotic Gene Expression by Transcriptional Attenuation

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The regulation of transcriptional elongation and termination is well documented as a means of controlling the expression of prokaryotic genes, and molecular and genetic analysis has yielded a clear understanding of the diverse mechanisms involved (reviewed in Yanofsky, 1988; Spencer and Groudine, 1990; Kerppola and Kane, 1991). It is now evident that the expression of several eukaryotic viral and cellular genes is also regulated at the stage of transcriptional elongation rather than at the more classically recognized initiation phase; examples include the proto-oncogenes c-myc and c-fos, a number of constitutively expressed and heat shock-inducible genes in *Drosophila,* and the integrated genome of the human immunodeficiency virus (HIV). Recent studies have revealed common underlying mechanisms whereby transcriptional elongation may be used to modulate eukaryotic gene expression according to diverse physiological signals.

TRANSCRIPTIONAL ATTENUATION IN EUKARYOTIC DNA VIRUSES

The process by which transcriptional pausing and termination is used to modulate the expression of downstream sequences is commonly known as attenuation (Yanofsky, 1988). The first demonstrations of transcriptional attenuation in eukaryotes emerged from analysis of RNAs expressed from the SV40- and adeno-viruses in infected animal cells (Laub *et al.,* 1980; Hay *et al.,* 1982; Aloni and Hay, 1985). SV40 late transcription was shown to terminate prematurely at a site 95 nucleotides (nt) downstream from the promoter when analyzed in either isolated nuclei or SV40 viral transcription complexes. Nucleotide sequences at the site of premature termination comprise dyad symmetries such that the corresponding RNA can be folded into alternative conformations consisting of either one or two stem-loop structures. It was suggested that these structures mediate transcriptional readthrough and attenuation, respectively, and that late gene expression is modulated by the viral-encoded agnoprotein via stabilization of the attenuation conformation. Sites of transcriptional pausing or premature termination have been shown in several other DNA viruses, notably adenovirus (Evans *et al.,* 1979; Maderious and Chen-Kiang, 1984; Mok *et al.,* 1984), polyoma (Skarnes and Acheson, 1991), and minute virus of mouse (Krauskopf *et al.,* 1991). Sites of premature termination are always located within several hundred nucleotides of the transcription start site but do not share common sequence features and are not all associated with regions of secondary structure in the corresponding RNA. In the case of adenovirus, premature termination has been reproduced upon in vitro transcription of cloned templates in HeLa cell extracts (Hawley and Roeder, 1985; Wiest and Hawley, 1990; Wiest *et al.,* 1992). In the presence of sarkosyl, transcripts terminated 186 nt downstream from the start site. Sequences required for premature termination resided within a 65-base pair (bp) region that functioned in an orientation-dependent manner when positioned at varying distances downstream from the adenovirus major late promoter but not when placed downstream from a heterologous globin promoter, and there was no evidence of RNA secondary structure within these sequences. The mechanisms that regulate use of attenuation signals in adeno- and other DNA viruses remain to be elucidated. Kessler *et al.* (1991) have suggested the existence of at least two types of viral attenuator; one class is dependent on RNA secondary structure and is exemplified by the SV40 late region attenuator, and a second class is sequence specific and modulated in part by the general transcription factors.

REGULATION OF TRANSCRIPTIONAL ELONGATION WITHIN EUKARYOTIC CELLULAR GENES

The regulation of transcriptional elongation has more recently come into focus as a means of modulating the expression of eukaryotic cellular genes, with the first example being found in c-myc. Measurement of the relative transcription across different regions of the human c-myc gene in the promyelocytic cell line, HL60, was achieved by hybridization of labeled nuclear runoff transcripts to filter bound probes corresponding to various parts of the gene. This revealed approximately threefold higher levels of transcription across the 5′ end of the gene as compared with the 3′ end and indicated a region of transcriptional elongation blockage close to the exon 1-intron 1 boundary (Bentley and Groudine, 1986). The reduction in steady-state levels of c-myc

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RNA observed upon differentiation or growth arrest of a variety of cell types was shown to result from a reduction in number of polymerases reading through this region, rather than from a reduction in transcriptional initiation (Bentley and Groudine, 1986; Eick and Bornkamm, 1986; Eick *et al.*, 1987; Nepveu *et al.*, 1987a,b). It was not possible to show whether the "elongation blockage" represented transcriptional pausing or a true termination event, because prematurely terminated transcripts with 3′ ends located within this region were not readily detectable in steady-state RNA of mammalian cells. Stable prematurely terminated RNAs were, however, detected upon transcription of cloned c-myc genes in microinjected *Xenopus* oocytes (Bentley and Groudine, 1988). The 3′ ends of prematurely terminated mouse c-myc transcripts mapped to a thymidine tract near the end of exon 1, whereas for the human gene, the sites mapped to two such tracts, one on either side of the exon 1-intron 1 boundary. In both human and mouse c-myc genes, the site of premature termination is preceded by sequences capable of forming a stable stem-loop structure in the corresponding RNA (Bentley and Groudine, 1988; Wright and Bishop, 1989). Such features resemble prokaryotic rho-independent intrinsic terminators, although a formal requirement for RNA secondary structure in premature transcriptional termination in the c-myc gene remains to be shown. DNA fragments encompassing the regions of premature termination mediated an elongation block when placed within a heterologous gene and assayed either in *Xenopus* oocytes or transfected mammalian cells (Bentley and Groudine, 1988; Miller *et al.*, 1989; Wright and Bishop, 1989). In addition, the transcription of cloned c-myc genes in in vitro systems indicated the presence of specific termination signals within these sequences. Using an assay in which purified RNA polymerase II initiated transcription from the end of dC-tailed templates containing c-myc segments, Kerppola and Kane (1988) showed that transcriptional pausing and premature termination took place within the region of the exon 1-intron 1 boundary; termination occurred irrespective of whether the transcript remained hybridized to or was displaced from the template, providing evidence against a requirement for RNA secondary structure. The low (7%) efficiency of intrinsic termination by purified pol II suggested that accessory components in the transcription machinery modulated termination in vivo. Transcriptional termination by purified RNA polymerase II always occurred within T-rich sequences, although not all T runs functioned as terminators, and the efficiency of termination did not correlate with the length of the T run (Kerppola and Kane, 1990). It was suggested that a structural element causing a bend in the DNA may be part of the signal for termination in this system. Transcriptional pausing at specific DNA sequences was greater in a system in which nascent RNA chains were elongated on purified RNA polymerase II transcription complexes after chromatin reconstitution (Izban and Luse, 1991). Premature transcriptional termination on c-myc templates was also observed in in vitro transcription reactions using HeLa cell nuclear extracts (London *et al.*, 1991), with the degree of termination depending on the growth conditions of the HeLa cells from which the extracts were prepared. Mixing experiments suggested that this reflected the relative amounts of termination or antitermination factors present; such factors could be involved specifically in myc transcription or represent general transcription elongation factors. Premature transcriptional termination in HeLa extracts was most efficient at relatively high salt concentrations (200 mM KCl). This was interpreted as a requirement for the dissociation of general transcription elongation factors that were presumed to be present in a greater excess over transcription complexes as compared to in vivo.

Blocks to transcriptional elongation and sites of premature termination have been detected in a number of other genes by nuclear runoff transcription analysis or the expression of cloned genes in *Xenopus* oocytes and in vitro transcription systems; in some cases the degree of attenuation is modulated according to various physiological signals. Examples include c-fos (Collart *et al.*, 1991; London *et al.*, 1991; Mechti *et al.*, 1991), c-myb (Bender *et al.*, 1987; Watson, 1988), adenosine deaminase (ADA) (Chinsky *et al.*, 1989; Lattier *et al.*, 1989; Chen *et al.*, 1990, 1991; Ramamurthy *et al.*, 1990), and tubulin (Middleton and Morgan, 1990). An additional site of premature transcriptional termination has recently been defined between the two promoters, P1 and P2, of the c-myc gene (Wright *et al.*, 1991; Meulia *et al.*, 1992; Roberts *et al.*, 1992). This site mediates premature termination of P1-initiated transcripts within the P2 promoter and may serve to modulate promoter usage in normal and tumor cells. As shown for c-myc, some attenuation sites have RNA secondary structures characteristic of prokaryotic rho-independent terminators, although many do not share such features or other sequence similarities. It has been suggested that the high C:G ratio in the RNA at the site of premature termination in the ADA gene resembles features recognized by the prokaryotic termination factor, rho, although this is not characteristic of other sites of attenuation (Innis and Kellems, 1991).

The causes of elongation blockages and premature termination events in the transcriptional of eukaryotic genes are not fully understood. Although many such regions are recognized to some extent by purified RNA polymerase II in the absence of accessory factors, it remains possible that pausing or termination is enhanced by the interaction of specific proteins with either DNA or RNA at these sites. G-rich elements are found at sites of premature termination between the P1 and P2 promoters and within exon 1 of the mouse c-myc gene (Wright *et al.*, 1991). It has been suggested that a common factor(s) may bind to these regions (Bossone *et al.*, 1992) and also to the G-rich element located at the site

of transcriptional termination between the closely linked complement genes, C2 and Factor B, in the major histocompatibility complex class III gene cluster (Ashfield *et al.*, 1991). In addition, a DNA-binding factor, TBF1, has been implicated in transcriptional termination of P1-initiated transcripts within the P2 promoter (Roberts *et al.*, 1992). In such cases of transcriptional termination within an adjacent promoter, it is possible that proteins binding within these regions may influence processes of both termination and transcriptional initiation. This has been implicated in the transcription of the ribosomal RNA genes by RNA polymerase I (McStay and Reeder, 1990b) and in adenovirus, where binding of the CCAAT box protein to the major late promoter possibly mediates termination of upstream transcripts and thus prevents transcriptional interference (Connelly and Manley, 1989a,b). Such proteins could inhibit transcriptional elongation by a "roadblock" type mechanism as has been characterized in some prokaryotic systems (reviewed in Spencer and Groudine, 1990). No DNA binding proteins have so far been implicated in general transcriptional termination at sites downstream from pol II–transcribed genes, although a well-characterized DNA binding activity mediates termination by eukaryotic RNA polymerase I (Kuhn *et al.*, 1990; McStay and Reeder, 1990a; Pfleiderer *et al.*, 1990; Smid *et al.*, 1992). Similarly, no proteins have been shown to interact with RNA sequences at sites of premature termination. Such proteins could function in a manner analogous to the prokaryotic termination factor rho, which binds to an unstructured C-rich region of RNA (Platt, 1986; Alifano *et al.*, 1991; Hart and Roberts, 1991; Richardson, 1991; Rivellini, *et al.*, 1991), or the mammalian protein La, which binds uridylate residues in the RNA at sites of termination by eukaryotic RNA polymerase III (Gottlieb and Steitz, 1989a,b). There is contradictory evidence concerning the actual number of sites of transcriptional attenuation that may be found within any given eukaryotic gene. Deletion of specific regions from the site of attenuation within exon 1 of the c-myc gene has resulted either in transcriptional readthrough (Miller *et al.*, 1989) or in attenuation at other downstream sequences (Krumm *et al.*, 1992).

The factors and recognition elements that modulate readthrough of elongation blockages and premature termination signals within eukaryotic genes are as yet uncharacterized. Diverse types of regulatory element control transcriptional elongation through discrete termination signals in prokaryotes. Different antiterminator proteins interact with either DNA or RNA at sites within the promoter, near the terminator (Houman *et al.*, 1990; Amster-Choder and Wright, 1992), or located in between (reviewed in Roberts, 1993). In contrast, few regulatory elements that control the use of transcription termination signals, either within or at the 3′ end of eukaryotic genes, have been defined. Examples include recognition of termination signals at the 3′ end of the U2 snRNA gene by transcripts initiating only from the corresponding snRNA gene promoter (Neuman de Vegvar *et al.*, 1986; Hernandez and Lucito, 1988), and the regulation of transcriptional elongation within the HIV genome by interaction of the transactivating factor TAT with RNA in the viral TAR region (to be discussed later). The general eukaryotic transcription factors that play a role in elongation include TFIIX, TFIIF, and TFIIS (see Kassavetis and Geidushek, 1993 and references therein). TFIIF is required at both initiation and elongation stages of transcription and increases RNA chain elongation relatively uniformly. In contrast, TFIIS associates with the transcription complex after initiation, enabling it to overcome intrinsic sites of pausing and premature termination in vitro (Reines *et al.*, 1989; SivaRamen *et al.*, 1990; Bengal *et al.*, 1991) and also to read through blocks to elongation caused by bound protein complexes. TFIIS has been shown to enhance the 3′ to 5′ hydrolytic activity of RNA polymerase II (Izban and Luse, 1992; Reines, 1992) with this activity being essential for TFIIS mediated readthrough of pause sites. Factors that regulate transcriptional elongation within specific genes could function via interaction with general transcription elongation factors in a manner analogous to the interaction of eukaryotic enhancer DNA-binding proteins with the general transcription initiation factors TFIIB or TFIID (Greenblatt, 1991).

Recent evidence indicates that eukaryotic cellular genes may be transcribed by more than one class of elongation complex. The drug 5,6-dichloro-1b-D-furanosylbenzimidazole (DRB) was shown to inhibit production of transcripts elongating past the site of premature transcriptional termination in the c-myc gene in microinjected *Xenopus* oocytes; DRB had no effect on production of prematurely terminated transcripts, thus revealing two classes of transcription complex (Roberts and Bentley, 1992). Similarly, by analyzing transcriptional elongation on cloned templates in *Drosophila* cell extracts, Marshall and Price (1992) have identified two classes of elongation complex. Productive elongation complexes generating full length transcripts were shown to derive from early paused complexes by the action of a factor called P-TEF, which is inhibited by DRB (Kephart *et al.*, 1992; Marshall and Price, 1992). It was suggested that regulatory proteins could influence transcriptional elongation by enhancing the activity of P-TEF or a similar cellular factor.

PROMOTER PROXIMAL PAUSING OF TRANSCRIPTION COMPLEXES IN EUKARYOTIC GENES

Recent experiments have drawn attention to the role of promoter proximal pausing of transcription complexes in regulation of transcriptional elongation. Lis and colleagues have suggested that for a number of *Drosophila* genes the ability of RNA polymerase II to elongate transcription through a gene may be specified while being held in a paused complex near the promoter. It was

shown by in vivo UV-crosslinking that RNA polymerase II is associated with the promoter of the *Drosophila* hsp70 gene before heat induction (Rougvie and Lis, 1990; Gilmour *et al.*, 1991; O'Brien and Lis, 1991). This polymerase was paused after the synthesis of ~25 nt and upon heat shock elongated through the rest of the gene. Such paused polymerases were shown to be present on a number of constitutively expressed and heat shock–inducible genes, with resumption of elongation by paused polymerase being the rate-limiting and hence regulable step in gene transcription. Paused polymerases were not present on the yolk protein gene, YP1, that is never expressed in the male tissues examined. Lee *et al.* (1992) have defined a region within the hsp 70 promoter that binds a constitutively expressed factor and plays a role in generating the paused complex; formation of a paused complex was a prerequisite for induction of the gene. This indicated that certain factors are responsible for formation of paused polymerase complexes on appropriate genes, with others being involved in resumption of transcriptional elongation.

Another approach that has been used to map paused polymerase complexes within transcription units has been to detect the resulting regions of single-stranded DNA by virtue of their sensitivity to potassium permanganate modification. This method has been used to map open complexes on the promoters of prokaryotic and eukaryotic genes (Sasse-Dwight and Gralla, 1989; Kainz and Roberts, 1992; Wang *et al.*, 1992) and to detect paused polymerase complexes at the 5′ ends of *Drosophila* genes (Giardina *et al.*, 1992). Using a combination of permanganate treatment and in vivo genomic sequencing, Mirkovitch and Darnell (1992) showed that induction of transcription of the interferon-stimulated gene, ISG54, resulted in an increased permanganate sensitivity downstream from the transcription start site that was associated with a homogenous distribution of polymerases along the 282 nt analyzed. No regions of sensitivity were detected when the gene was not expressed in uninduced cells (Mirkovitch and Darnell, 1992). In contrast, expression of the transthretin gene in liver was associated with an increased permanganate sensitivity within specific sequences located 30 nt downstream from the promoter, indicating the presence of a paused polymerase. Similarly, Krumm *et al.* (1992) detected paused polymerases downstream from the promoter of the c-myc gene and suggested that this may represent a site for modification of the transcription complex so as to control its elongation properties throughout the rest of the gene. By performing nuclear runoff transcriptions using very short probes, Strobl and Eick (1992) have confirmed the hold-back of RNA polymerases at the promoter of the c-myc gene. No examples of paused transcription complexes on nonexpressed mammalian genes have been found, and, in contrast to the *Drosophila* genes, there is no evidence as yet to suggest that actual release from a paused complex represents a rate-limiting, regulable step in transcription. Modification of polymerase at the pause site could, however, regulate downstream rate-limiting processes, e.g., transcriptional elongation through termination signals. This is therefore reminiscent of the mechanism of action of the phage λ antitermination protein, Q, that is required for expression of the λ late genes. Q modifies RNA polymerase while being held during an extended pause in elongation at +17 of the late promoter transcript and interacts with specific DNA sequences at −10 to −35 of the promoter (Grayhack *et al.*, 1985; Goliger and Roberts, 1989; Goliger *et al.*, 1989; Yang and Roberts, 1989; Yang *et al.*, 1989; Yarnell and Roberts, 1992). Q-modified polymerase has altered elongation properties, pauses less efficiently, and transcribes through downstream termination signals into the λ late genes. The modification of eukaryotic transcription complexes in promoter proximal pauses remains to be proven, and regulatory proteins interacting with paused complexes have yet to be identified. Such proteins may be expected to have somewhat different functions from Q, in that they modulate earlier events in transcriptional elongation, e.g., exit from stalled complexes at the promoter or transit through intrinsic pause sites and termination sites near the 5′ end of the gene.

REGULATION OF TRANSCRIPTIONAL ELONGATION IN HIV EXPRESSION

The only example of modulated transcriptional elongation in eukaryotes in which both a regulatory protein and its target of interaction have been defined is found in HIV. The viral encoded transactivating factor, TAT, is required for expression of the integrated proviral DNA and acts partly by regulating the elongation of transcripts initiating from the long terminal repeat (LTR) promoter (reviewed in Cullen, 1990, 1991; Karn, 1991, 1992; Frankel, 1992). TAT protein interacts with the RNA product of the transactivation response region (TAR), which is located at residues +1 to +79 with respect to the LTR promoter and which folds into a highly stable stem-loop RNA structure (Dingwall *et al.*, 1989, 1990). A U-rich bulge located just below the loop is important for TAT recognition. It has been suggested that TAT may regulate both transcriptional initiation and elongation with Kao *et al.* (1987) being the first to demonstrate that it activates HIV transcription by behaving as an antiterminator like protein. By analyzing the expression of an HIV LTR-driven CAT gene in transient expression assays, it was shown that transcripts did not elongate past the viral TAR region in the absence of a cotransfected TAT gene; this was indicated both by runoff transcription analysis and by the detection of truncated RNAs in the cytoplasm. In the presence of TAT, transcriptional elongation proceeded through to the end of the CAT gene; there was no effect of TAT on transcriptional initiation from the viral LTR in the system. The antitermination function of TAT thus re-

sembled that of the bacteriophage λ antitermination protein N, which interacts with an RNA stem-loop structure in the box B region of the transcribed λ *nut* site; this interaction results in the formation of a modified transcription complex that persists through downstream RNA synthesis and is now capable of reading through discrete transcription termination signals located many kilobases downstream (Barik *et al.*, 1987; Horwitz *et al.*, 1987; Lazinski *et al.*, 1989; Whalen and Das, 1990; Ghosh *et al.*, 1991; Das, 1992; Roberts, 1993). Part of the role of the phage N protein may be to enhance the otherwise weak binding of host bacterial antitermination proteins to an adjacent region, box A of the λ *nut* site, thus bringing the host antitermination system to the phage genome. It remains possible that TAT modifies eukaryotic transcription complexes in an analogous manner by altering the binding or activity of host cell proteins that regulate transcriptional elongation in eukaryotic cellular genes. Both N and TAT proteins have an arginine-rich subdomain that is required for RNA recognition (Lazinski *et al.*, 1989). Host cell proteins have been shown to interact with the TAR region of HIV, although their functions remain to be defined (Gatignol *et al.*, 1989; Marciniak *et al.*, 1990; Jeyapaul *et al.*, 1991; Sheline *et al.*, 1991; Shibuya *et al.*, 1992). There are, however, clear differences between the TAT- and N-mediated antitermination processes. The λ *nut* site will function as a discrete element when moved away from its cognate promoter, whereas TAT-mediated antitermination requires TAR to be located immediately adjacent to the HIV promoter (Selby *et al.*, 1989), and it also remains unclear whether TAT acts to overcome specific termination signals in the HIV TAR region in a manner analogous to the antitermination of the prokaryotic terminators. Most of the recent evidence suggests that in the absence of TAT, LTR-initiated transcription terminates at many, possibly random, downstream sites (Lapsia *et al.*, 1989; Marciniak and Sharp, 1991; Kessler and Mathew, 1992). Marciniak and Sharp (1991) have suggested that in the absence of TAT, the distribution of RNA polymerases along an HIV LTR-driven gene in an in vitro system is such as would be predicted by the existence of two classes of elongation complex initiating from the HIV promoter. Most complexes are of the less processive unstable form and terminate transcription at a relatively uniform rate over the first 1000 bp of template; the less abundant form is more processive and stable and elongates through the several kilobases of template analyzed. TAT was suggested to enhance transcriptional elongation by causing ~10-fold relative increase in the more processive class of complex. Evidence for the presence of two distinct classes of complex transcribing from the HIV LTR in vitro was also provided by the effect of the drug DRB, which inhibited production of the stabilized elongation-competent transcription complexes formed in the presence of TAT but had no effect on production of the less processive complexes. This is similar to the finding of different classes of transcription elongation complex on eukaryotic cellular genes, and Marshall and Price have suggested that the role of TAT may be to enhance the activity of a cellular factor, such as P-TEF, which is required for formation of processive transcription elongation complexes on host genes. Other evidence suggests that TAR sequences themselves may act as a specific transcriptional terminator in vitro (Bengal and Aloni, 1991), and it has been shown that TAT-modified transcription complexes are capable of reading through a specific terminator derived from the U2 snRNA gene in artificial constructs in vivo (Ratnasabapathy *et al.*, 1990). TAT may therefore function both by increasing the general processivity and stability of transcriptional elongation complexes and by enabling such complexes to read through specific termination signals.

CONCLUSIONS

The regulation of transcriptional elongation is now recognized as an important component in the control of eukaryotic gene expression. Most evidence suggests that various classes of elongating transcription complex differ in their ability to proceed beyond the 5′ end of the gene, with regions of elongation blockage being manifest as paused complexes at the promoter or premature termination events near the 5′ end of the gene. Factors that regulate transcription elongation complexes and that control transcriptional attenuation in individual genes according to different physiological signals remain to be defined. Such factors could function via interaction with ubiquitous proteins that play a role in transcriptional elongation and could represent previously identified transcription factors.

The use of transcriptional elongation as a rate-limiting step in the expression of certain eukaryotic genes ensures that the gene is expressed only when appropriate factors or activities that control this stage of transcription are present. Thus, transcription from the HIV LTR does not elongate beyond a few hundred nucleotides in the absence of TAT, even though the HIV promoter contains multiple binding sites for known transcriptional activators, notably NFκB and SP1. Host proteins that control the elongation of polymerase in eukaryotic cellular genes may play a similar role in ensuring that a gene is expressed only when appropriate, even in the presence of other types of transcriptional activator bound to the promoter. This mechanism may thus play a biological role in coordinating responses to different physiological signals. It remains to be determined whether transcriptional attenuation is most commonly found in the regulation of genes in which rapid responses to external signals is continuously required, e.g., heat shock genes in *Drosophila* or the c-myc gene in mammals, rather than in the regulation of genes after more permanent activation or repression during differentiation and development.

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