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Cell-cycle-specific transcription termination within the human histone H3.3 gene is correlated with specific protein–DNA interactions

ALAN TAYLOR[1]*, LIQUN ZHANG[2], JOHN HERRMANN[2], BEI WU[2], LARRY KEDES[3] AND DAN WELLS[2]

[1] *Molecular Biology Core Laboratory, Department of Biological Sciences, Wichita State University, 1845 Fairmount, Wichita, KS 67260–0026, USA*
[2] *Institute for Molecular Biology and Department of Biology, University of Houston, Houston, TX 77204–5513, USA*
[3] *Department of Biochemistry, University of Southern California, School of Medicine, Los Angeles, CA 94304, USA*

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Summary

In vitro studies using highly purified calf thymus RNA polymerase II and a fragment spanning the first intron of H3.3 as template DNA have demonstrated the existence of a strong transcription termination site consisting of thymidine stretches. In this study, nuclear run-on experiments have been performed to assess the extent to which transcription elongation is blocked *in vivo* using DNA probes corresponding to regions 5′ and 3′ of the *in vitro* termination sites. These studies suggest that H3.3 expression is stimulated following the inhibition of DNA synthesis through the elimination of the transcription elongation block. Interestingly, both the *in vivo* and *in vitro* experiments have revealed that the transcriptional block/termination sites are positioned immediately downstream of a 73 bp region that has been over 90% conserved between the chicken and human H3.3 genes. The extreme conservation of this intronic region suggests a possible role in maintaining *cis*-acting function. Electrophoretic mobility shift experiments show that HeLa cell nuclear extracts contain protein factors that bind specifically to the region of transcription elongation block. Furthermore, we demonstrate a correlation between the protein binding activity and the transcriptional block in cells that have been either arrested at the initiation of S phase or were replication-interrupted by hydroxyurea. DNA footprinting experiments indicate that the region of protein binding is at the 3′ end of the conserved region and overlaps with one of the three *in-vitro*-mapped termination sites.

1. Introduction

The control of transcription elongation and termination by RNA polymerase II are important regulatory steps in eukaryotic gene expression. Examples of regulation at the elongation phase are well documented in both eukaryotic and prokaryotic systems (Bender *et al.*, 1987; Chinsky *et al.*, 1989; Lattier *et al.*, 1989; Spencer & Groudine, 1990; Kerppola & Kane, 1990; Reines, 1992; Wright, 1993). Elongation by RNA polymerase II in these genes conditionally pauses and in some cases leads to premature termination. As examples, the human and murine c-*myc* (Bently & Groudine, 1986; Eick & Bornkamm, 1986; Mechti *et al.*, 1986; Collart *et al.*, 1991; Strobl & Eick, 1992) and multiple *Drosophila* heat shock genes (Rassmussen & Lis, 1993; Rassmussen & Lis, 1995) are down-regulated due to a block in elongation. In these genes the elongation pause site is relatively close to the transcription initiation site. It has been suggested that the paused RNA polymerase II may be 'tethered' to the initiation complex or that the polymerase may encounter an obstacle such as an abnormal DNA structure or DNA-bound protein (Rassmussen & Lis, 1995).

Reines *et al.* (1987) have demonstrated the presence of an *in vitro* transcription termination site within the first intron of the human H3.3 gene that promotes the premature release of elongating transcripts. Mapping of the 3′ ends of these truncated transcripts indicated that they all terminate within one of three consecutive stretches of 5–8 thymidylate residues in the non-transcribed strand. Similar T runs were found to have no effect on RNA polymerase II elongation, suggesting

* Corresponding author. Tel: +1 (316) 978–3111. Fax: +1 (316) 978–3772. E-mail: mtaylor@twsuvm.uc.twsu.edu.

that surrounding sequences and/or *trans*-acting factors may play an important role in H3.3 transcriptional attenuation. It has also been demonstrated that the elongation factor SII allows readthrough of the termination sites within H3.3 *in vitro* (Reines *et al.*, 1989). A bend in the DNA helix which occurs in the region of transcription termination has been identified as an integral component of the termination signal for polymerase II within the intron of the H3.3 gene (Kerppola & Kane, 1990; Reines, 1992).

Genes regulated through control of transcription elongation should as a consequence possess an uneven distribution of RNA polymerase. In the present study we use a nuclear run-on assay to demonstrate that the *in vitro* transcription termination observed previously within the human H3.3 histone gene (Reines *et al.*, 1989) also occurs *in vivo*. This block to transcription elongation is located within the first intron and immediately follows a 73 bp sequence which is over 90% conserved between chickens and humans. We demonstrate that this transcriptional block is rapidly abolished both in HeLa cells treated with 5 mM hydroxyurea to inhibit DNA synthesis and in HeLa cells arrested at the beginning of the S phase of the cell cycle by a thymidine/aphidicolin block. An electrophoretic mobility shift assay (EMSA) was employed to demonstrate DNA binding proteins in crude HeLa cell nuclear extracts that interact specifically with a DNA fragment spanning the conserved sequence and the region of the transcriptional termination sites. We have correlated this DNA–protein interaction with the block in trancription elongation and shown that the region of protein binding overlaps with one of the three *in-vitro*-mapped termination sites at the 3′ end of the conserved region.

2. Materials and methods

(i) *Sequence analysis*

Sequencing was performed using the dideoxy method of Sanger *et al.* (1977) after subcloning of restriction fragments into M13 vectors. The entire nucleotide sequence shown in Fig. 1 was determined multiple times in both orientations. Sequence data were managed by the IntelliGenetics program GEL. The genome sequence data base (GSDB) was screened using the FASTA program on the Q server.

(ii) *Nuclear run-on analysis*

Reactions to label nascent RNAs were performed essentially as described by Greenberg & Ziff (1984) with slight modifications. Cells were washed in phosphate-buffered saline (PBS) at 4 °C and collected in plastic centrifuge tubes. Pellets containing about 10^8 cells were resuspended in hypotonic buffer (20 mM Tris pH 8·0, 4 mM $MgCl_2$, 6 mM $CaCl_2$). After a few minutes on ice, an equal volume of 0·6 M sucrose and 0·2% NP-40 was added and cells were lysed by gentle vortexing. Nuclei were collected by centrifugation at 500 *g* for 2 min. Nuclei were resuspended in 5 ml of resuspension buffer (0·25 M sucrose, 10 mM Tris pH 8·0, 10 mM $MgCl_2$, 0·5 mM dithiothreitol (DDT), counted and checked for complete cell lysis. Nuclei were again collected by centrifugation and resuspended into 2 × reaction buffer (Greenberg & Ziff, 1984) and 50% glycerol at a concentration of $\sim 10^7$ nuclei/150 μl for freezing. Nuclei were then frozen at −80 °C until use. For run-on RNA synthesis, 150 μl of nuclei was mixed with 150 μl of 1 × reaction buffer containing 0·25 mCi of [^{32}P]UTP (800 Ci/mM) and 60 U of RNAsin and incubated at 26 °C for 30 min. Following the reaction, DNase I was added to a concentration of 10 mg/ml and incubated for 10 min at 26 °C. The reaction was then made 1·0% SDS, 0·1 mg/ml tRNA, 10 mM Tris 7·0, 5 mM EDTA and 200 μg/ml proteinase K and incubated for 30 min at 37 °C. The mixture was then phenol extracted and the radiolabelled RNA was ethanol precipitated.

(iii) *Nuclear extract preparation*

Exponentially growing HeLa S3 cells were washed in PBS at 4 °C. The PBS was removed and 1 ml of a hypotonic buffer (20 mM Tris-HCl, pH 8·0, 4 mM $MgCl_2$, 6 mM $CaCl_2$ and 0·5 mM DTT) was added per 100 mm diameter plate for 5 min. One millilitre of lysis buffer (0·6 M sucrose, 0·2% Nonidet P40 and 0·5 mM DTT) was added and the cells were harvested. Following a 2 min low-speed (500 *g*) spin, the crude nuclear pellet was washed to remove residual cytoplasmic material in 0·25 M sucrose, 10 mM Tris-HCl pH 8·0, 10 mM $MgCl_2$ and 0·5 mM DTT. Nuclei were collected again at 500 *g* for 2 min. The nuclear pellet was resuspended in 20 mM HEPES, 20% w/v glycerol, 100 mM KCl, 0·5 mM phenylmethylsulphonylfluoride (PMSF), 0·5 mM DTT and 400 mM NaCl and incubated on ice for 1 h. The nuclei were collected by a brief centrifugation at 12000 *g* and the supernatant was assayed for total protein content using a coomassie-blue-based assay developed by BIO-RAD. Gamma-globulins were used as standards and extracts were usually found to contain 4–6 mg/ml of protein.

(iv) *Electrophoretic mobility shift assay*

All protein–DNA binding reactions were performed according to Sen & Baltimore (1986), in 10 mM Tris (pH 7·5), 40 mM NaCl, 1 mM EDTA and 4% glycerol for 20 min at room temperature. A typical binding reaction contained 1 μg poly(dI):poly(dC) (Pharmacia or Sigma) as non-specific competitor, 0·5–1 ng binding site DNA probe which had been end-labelled with ^{32}P to a specific activity of 50000 cpm/ng using T4 DNA polymerase, and 5–10 μg of nuclear extract (added last). The optimum quantity of poly(dI):poly(dC) was

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determined empirically for each nuclear protein sample. Complexed probe DNA was resolved from free DNA on a low ionic strength 6% polyacrylamide gel prepared according to Halligan & Desiderio (1987) in 33·5 mM Tris-HCl (pH 7·5), 16·5 mM sodium acetate and 5 mM EDTA. The gel was pre-run for 1 h in 0·5 × TBE at 100 V/cm and electrophoresis was at the same voltage. The gel was dried and the bands visualized by autoradiography.

(v) *Cell cycle synchronization*

The HeLa cells were synchronized by sequential thymidine and aphidicolin treatment (Heintz *et al.*, 1983). Before treatment, cells were grown in tissue culture flasks at 4×10^5/ml in Dulbecco's modified Eagle medium containing 5% newborn calf serum. Cells were initially treated with 2 mM thymidine for 12 h, released from the block by three washes with fresh medium without calf serum, and then grown in complete medium containing $0{\cdot}24 \times 10^{-4}$ M thymidine and deoxycytidine. After 9 h, aphidicolin was added to 5 μg/ml for an additional 12 h. This treatment arrests cells at the G_1/S border before initiation of S phase DNA synthesis. To allow cells to progress through S phase, the aphidicolin was washed out as above and cells were allowed to grow in fresh medium. The initiation of DNA synthesis was monitored by incorporation of [^{3}H]deoxycytidine (Nilsen & Baglioni, 1979). In synchronized cells grown alongside those used in the nuclear run-on assays, an increase in ^{3}[H]deoxycytidine incorporation was seen immediately (within 30 min) following release from aphidicolin and peaked at about 3 h following release.

(vi) *MPE footprinting*

MPE (methidiumpropyl-EDTA) is a DNA intercalator synthesized by Dyke & Dervan (1983). It cleaves double-stranded helical DNA in the presence of ferrous ions and oxygen. The cleavage is random, exhibiting no sequence specificity, and thus allows precise delineation of protection boundaries. The protein binding reaction is carried out as described for the EMSA, but with triple the reagents totalling 50 μl. Five microlitres of 0·1 mM MPE–Fe II was added to the mixture. The addition of 1 μl of 0·1 M DTT initiated the cleavage reaction, which was allowed to continue at 37 °C for 15 min. At this time the reaction was stopped by placing the tubes at −80 °C. Samples were then lyophilized and resuspended into 100 μl of 10 mM Tris buffer followed by phenol/chloroform extraction and ethanol precipitation. Formamide loading buffer was added to each sample and samples were electrophoresed on 8% polyacrylamide–8 M urea sequencing gels.

3. Results

(i) *A conserved DNA sequence immediately precedes* in vitro *termination sites*

On the basis of genomic blot hybridization analysis (Wells *et al.*, 1987), we have previously shown that the entire sequence contained within the first intron is present at only one site in the human genome. To characterize the entire region surrounding the *in vitro* transcription termination sites, the sequence of the first intron was determined and is presented in Fig. 1. This sequence represents 900 nucleotides (nt) of new sequence and 600 nt of previously published sequence (Wells & Kedes, 1985; Reines *et al.*, 1987; Wells *et al.*, 1987). The *in vitro* termination sites are indicated by black dots below the mapped thymidine residues.

The intron sequence surrounding the termination sites was compared with all sequences in the GSDB to identify other similar genomic sequences. Only one sequence showed any significant similarity to the intron sequence. The result of that sequence similarity search is shown in Fig. 2. A 71 nt region within human H3.3 histone intron 1 was shown to be 90·4% similar to a 73 nt region within the first intron of a chicken H3.3 gene (Brush *et al.*, 1985) allowing for one 2 nt gap. If the gap is disallowed, the two sequences are 92·4% similar over a 66 nt region. Except for this region, the two introns show no significant similarity. The region of similarity between these two evolutionarily related genes includes the first *in-vitro*-mapped transcription termination site.

(ii) *H3.3 transcription is blocked* in vivo *at the level of elongation*

In vivo nuclear run-on transcription assays were performed to analyse the intragenic transcription block previously identified *in vitro*. In these experiments, nuclei were purified from HeLa cells and nascent transcripts were elongated in the presence of [^{32}P]UTP. Following this elongation step, radiolabelled nascent RNA was isolated and hybridized to single-copy intron fragments corresponding to the regions immediately upstream and downstream of the *in vitro* termination sites. The upstream region (*Sac*I–*Xho*I fragment) and the downstream region (*Xho* I–*Nco* I fragment) were of equivalent size and G+C percentage (Fig. 3*A*). Assuming a constant elongation rate, the strength of the hybridization signals to different fragments of the intron directly relates to the number of RNA polymerase molecules actively engaged in transcription on the corresponding portion of the intron. The results of a representative experiment are shown in Fig. 3*B* and the results of four replicate experiments are summarized in Table 1. The data indicate that there is 69·5% (±4·4%, SD) decrease in transcriptional activity occurring in the downstream intron region (*Xho*I–*Nco*I fragment). The

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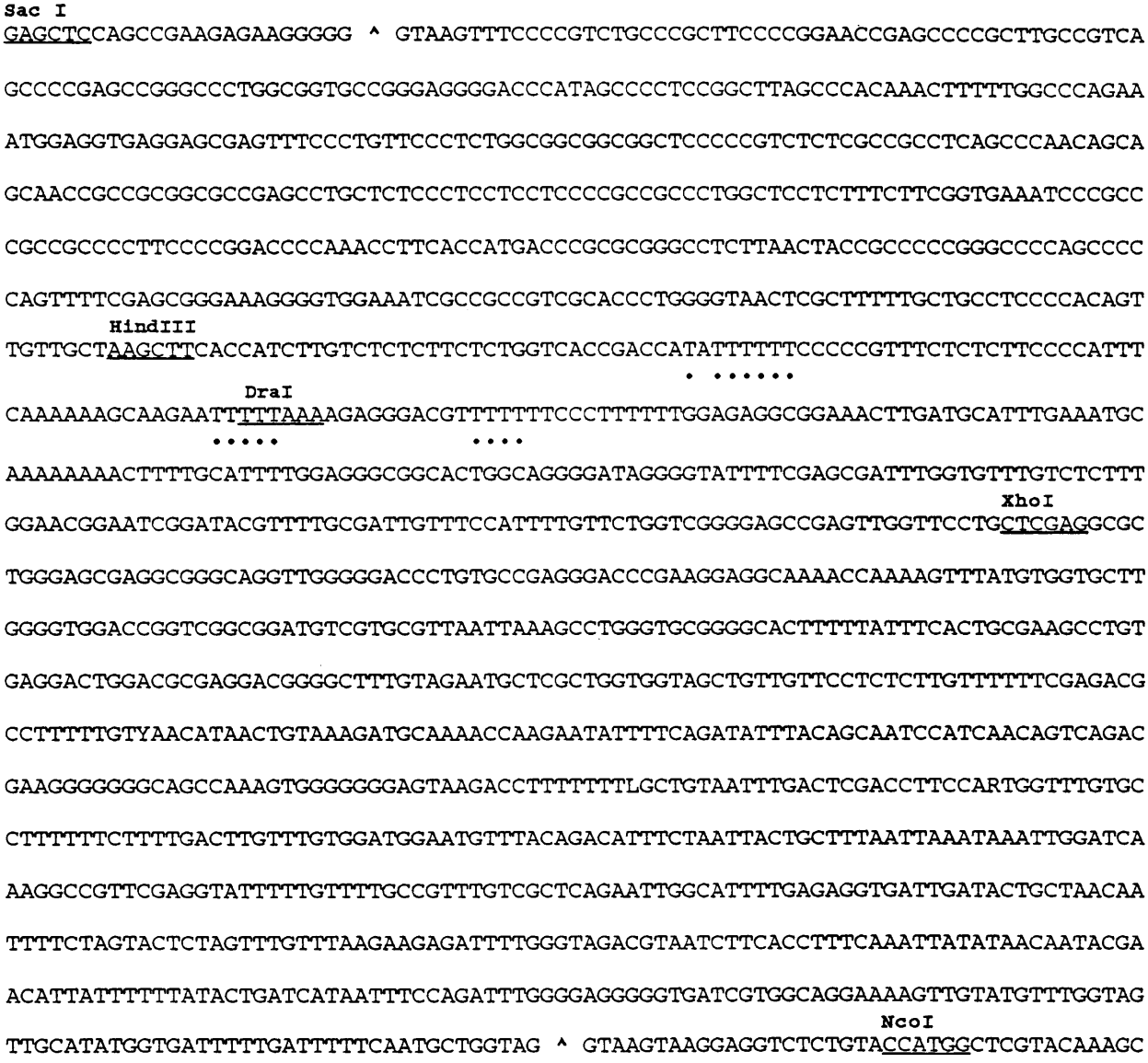

Fig. 1. Sequence of human H3.3 histone intron 1. The '^' symbol indicates the intron–exon junctions. The '.' symbol below the DNA sequence represents the RNA polymerase II termination sites mapped *in vitro*. Restriction enzyme sites referred to in the text are underlined and labelled.

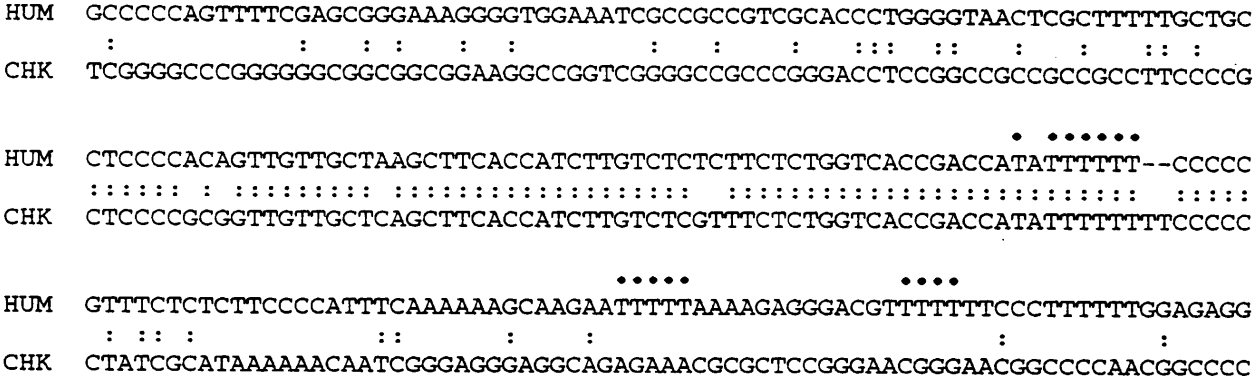

Fig. 2. Conserved region of the first intron of the human H3.3 gene. A region of the first intron of the human H3.3 gene (HUM) and a similar region of the chicken H3.3 gene (CHK) are aligned and compared. The '.' symbols indicate the RNA polymerase II termination sites mapped *in vitro*.

decreased transcriptional activity occurs downstream of the *in vitro* termination site and may include sequences within the *Xho*I–*Nco*I intron fragment. Control experiments were performed to show that the transcriptional activity was due to RNA polymerase II and could not be explained by opposite-strand

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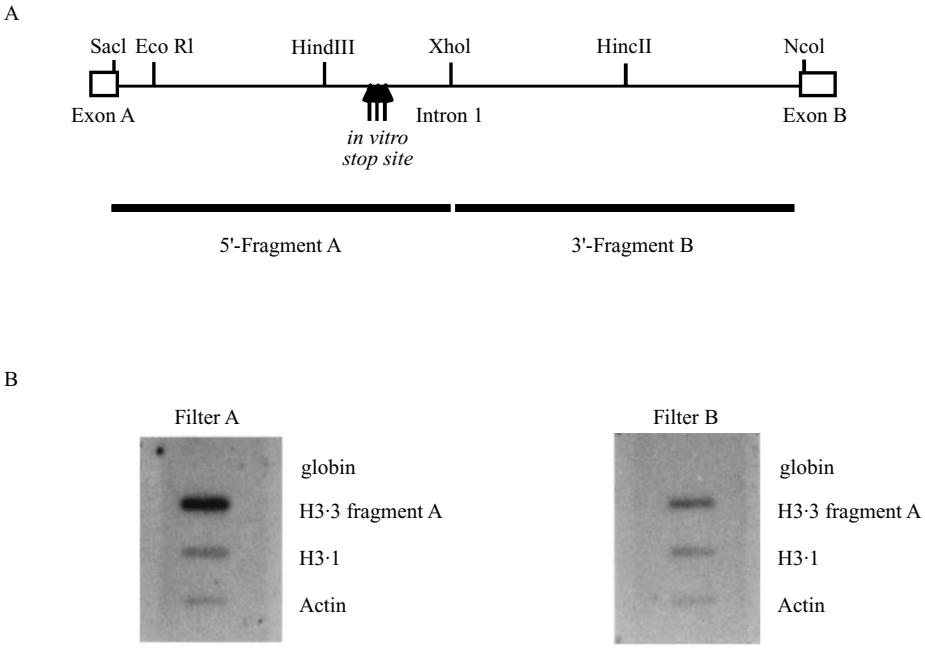

Fig. 3. Nuclear run-on analysis. (*A*) Physical map of the first intron of the human H3.3 gene indicating the location of *in vitro* termination sites and the restriction endonuclease cleavage sites. (*B*) Slot blot analysis of radiolabelled nascent RNA hybridized to various DNA fragments as described in the text. Globin, a 1 kb fragment containing the human alpha-globin gene; human H3.3 fragment A and B, described in 3A; H3.1, a 1.7 kb fragment of the H3.1 gene; Actin, a 500 bp fragment from the α-actin gene. Two micrograms of each gene fragment was blotted onto each slot. The nascent radiolabelled RNA was split into two equal aliquots and hybridized to filters A and B separately. The densitometric scans of the autoradiographs were integrated and the results are shown in Table 1. The data shown above are from experiment 2 (Table 1).

Table 1. *Densitometric quantitation of nuclear run-on data*

	H3.3 5′ fragment (A)	H3.3 3′ fragment (B)	H3.1[a]	Actin[a]
Experiment 1	100	26·7	17·1	14·6
Experiment 2	100	28·4	18·6	7·5
Experiment 3	100	38·0	28·4	26·5
Experiment 4	100	29·0	12·1	ND
Average	100	30·5	19·0	16·2

Autoradiographs from HeLa nuclear run-on experiments were analysed by densitometry and each autoradiograph was corrected for film background. The most intense hybridization signal in each experiment (H3.3 fragment A) was assigned a relative value of 100.
[a] The values for H3.1 and actin represent the averages of the two filters for each experiment. The statistical evaluation of the difference in hybridization signal between H3.3 fragment A and fragment B is discussed in the text.

transcription of antisense RNA (data not shown). These results strongly suggest that there is indeed an *in vivo* block to transcription elongation within the first intron and that the location of the block is consistent with the termination sites observed *in vitro*.

(iii) *The transcription elongation block is relieved by DNA synthesis inhibition*

Unlike the H3.3 gene, transcription of most vertebrate histone genes is under cell cycle regulation and the genes are transcribed only during S phase (Heintz *et al*., 1983). The transcription of these replication-dependent histone genes is negatively affected by DNA synthesis inhibition. To examine the effect of DNA synthesis inhibition on the replication-independent H3.3 gene, we performed nuclear run-on assays in the presence of a DNA synthesis inhibitor. In these experiments HeLa cells were treated for 0, 1 or 4 h with 5 mM hydroxyurea prior to the isolation of their nuclei. The radiolabelled nascent RNAs were hybridized to H3.3 intron fragments corresponding to

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Table 2. *Densitometric quantitation of nuclear run-on data: hydroxyurea-treated and synchronized HeLa cells*

	H3.3 fragment A	H3.3 fragment B
Untreated cells[4]	100	23·8
1 h HU treatment[3]	100	44·2
4 h HU treatment[3]	98·0	100
Thym/aphid (t/a) block[2]	100	88·3
0·5 h post t/a release[2]	100	21·6
3 h post t/a release[2]	100	9·2
6 h post t/a release[2]	100	35·1

Autoradiographs from nuclear run-on experiments were analysed by densitometry and each autoradiograph was corrected for film background. The most intense hybridization signal in each experiment (H3.3 fragment A) was assigned a relative value of 100. HU treatment indicates HeLa cells treated with 5 mM hydroxyurea to inhibit DNA synthesis. Thym/aphid block indicates HeLa cells blocked at the initiation of S phase by sequential thymidine and aphidicolin treatments (see Materials and Methods). The superscript numbers indicate that the data represent an average value from two ([2]), three ([3]), or four ([4]) separate experiments.

the region immediately upstream and downstream of the *in vitro* termination sites as described for Fig. 3*A*. The results of these experiments are tabulated in Table 2. As expected, the untreated controls showed about a fourfold decrease in the number of transcripts hybridizing to the fragment downstream of the termination sites (fragment B) as compared with the upstream fragment (fragment A). However, after only 1 h of hydroxyurea treatment, the difference was only about twofold, and after 4 h of treatment there were approximately equal numbers of transcripts hybridizing to the intron region upstream and downstream of the termination sites. These results demonstrate the surprising conclusion that the transcription block seen in the first intron seems to be abolished within 4 h after DNA synthesis is inhibited with hydroxyurea.

As a second approach to analyzing the dependence on cell cycle phase and DNA synthesis, we synchronized HeLa cells by sequential thymidine and aphidicolin blocks. This treatment blocks cells at the initiation of S phase. Release from the block allows the cells to immediately enter S phase. The results of nuclear run-on assays of the S-phase-blocked cells indicate that transcription elongation block is abolished in these cells, in that approximately equal amounts of hybridization was seen to each side of the transcription termination site (Table 2). However, immediately following release from the S phase block, the transcription elongation block reappears. The extent of the block seems to peak at about 3 h following release, coinciding with the peak in DNA synthesis as measured by [^{3}H]deoxycytidine incorporation (see Section 2).

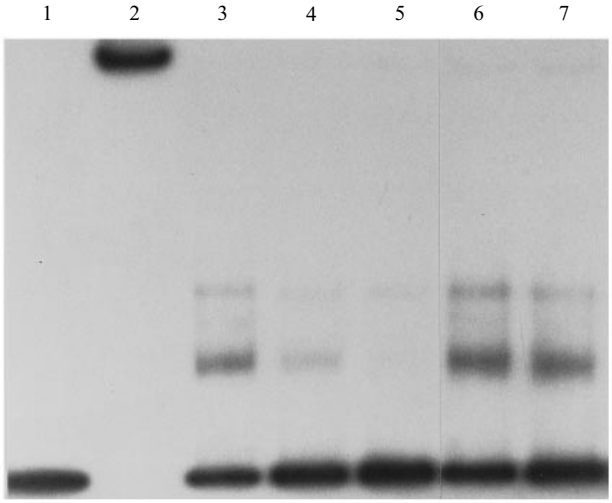

Fig. 4. Identification of HeLa nuclear factors binding to the H3.3 *Hin*dIII–*Xho*I intron subfragment by electrophoretic mobility shift assay. The 299 bp *Hin*dIII–*Xho*I fragment was labelled with ^{32}P using T4 DNA polymerase. The labelled fragment was incubated with HeLa nuclear extract followed by electrophoresis through a low ionic strength polyacrylamide gel as described in Sect. 2. To demonstrate that the observed binding was sequence specific, unlabelled *Hin*dIII–*Xho*I fragment from the H3.3 intron was used as a specific competitor in the binding reaction. Lane 1, 1 ng of end labelled *Hin*dIII–*Xho*I fragment; lane 2, labelled *Hin*dIII–*Xho*I fragment incubated with 15 μg of nuclear extract without poly dI:dC as a non-specific competitor. Lanes 3–7 all contain 1 ng of labelled *Hin*dIII–*Xho*I fragment, 5 μg of poly dI:dC and 15 μg of nuclear extract. Lanes 4 and 5 also contain 5 ng and 25 ng, respectively, of unlabelled *Hin*dIII–*Xho*I competitor fragment. Lanes 6 and 7 contain 5 ng and 25 ng, respectively, of a similar-sized unlabelled fragment from the CAT gene as a non-specific competitor.

(iv) *Specific binding of nuclear proteins to the region of the transcription elongation block*

The presence of an elongation block and transcription termination site within the first intron of the H3.3 gene indicates that an unusual mechanism for transcriptional regulation has evolved for the H3.3 gene. Fine mapping of the *in vitro* transcripts revealed that more than 66% are terminated within a series of 5–8 thymidylate residues in the non-transcribed strand (Reines *et al.*, 1987). The nature of these sequences suggested that transcript–template instability due to intrinsically weak dA:rU bonding could promote premature release of the elongating transcripts. However, this is unlikely to be the only factor involved at this termination site, since other similarly sized T-stretches were not seen to have any effect on RNA polymerase II release. To study the possibility that *trans*-acting nuclear factors could specifically interact with this region, EMSAs were employed.

EMSAs were performed using a 299 nt *Hin*dIII–*Xho*I fragment spanning the *in vitro* termination sites from H3.3 intron 1 (Fig. 1) incubated

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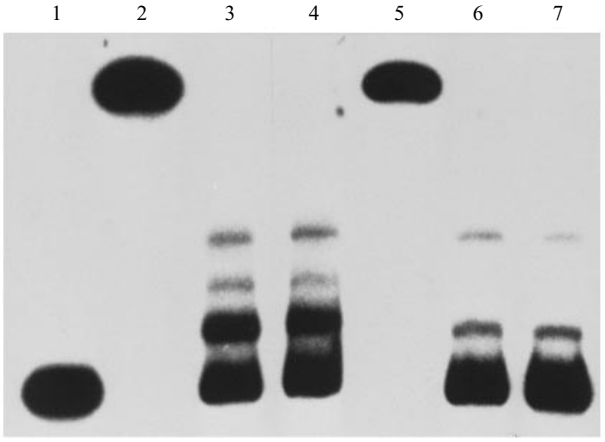

Fig. 5. The effect of hydroxyurea on the electrophoretic mobility shift assay. HeLa nuclear extract was prepared from control and hydroxyurea-treated cells as described in Sect. 2. Binding reactions utilized the 299 bp *Hin*dIII–*Xho*I fragment as labelled probe. Lane 1, 1 ng of labelled *Hin*dIII–*Xho*I fragment; lane 2, 1 ng of labelled *Hin*dIII–*Xho*I fragment plus 5 μg of untreated HeLa cell nuclear extract; lanes 3 and 4, 1 ng of labelled *Hin*dIII–*Xho*I fragment, 5 μg of untreated nuclear extract and 5 μg of poly dI:dC. Lane 5, 1 ng of labelled *Hin*dIII–*Xho*I fragment plus 5 μg of HeLa cell nuclear extract treated for 5 h with 5 mM hydroxyurea; lanes 6 and 7, same as lanes 3 and 4 except that the nuclear extracts were isolated from HeLa cells treated for 5 h with 5 mM hydroxyurea.

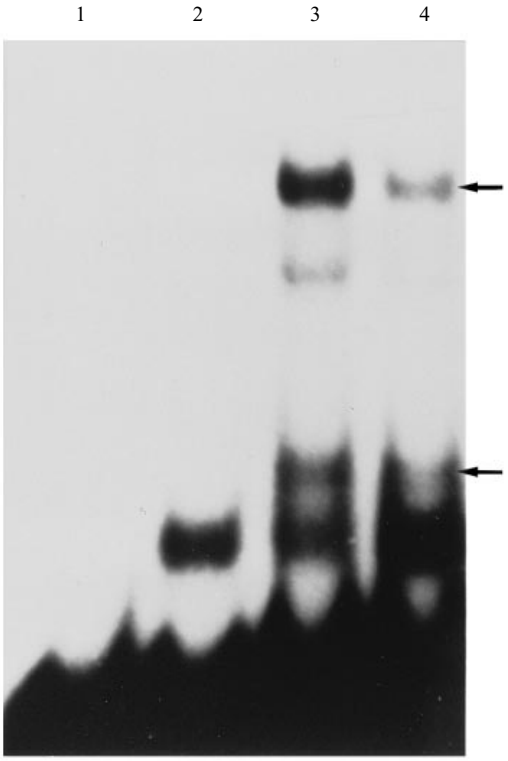

Fig. 6. The effect of cell cycle synchronization on the electrophoretic mobility shift assay. HeLa cells were blocked at the initiation of S phase by sequential thymidine and aphidicolin treatment (see Sect. 2). Binding reactions utilized 1 ng of the labelled 88 bp *Hin*dIII–*Dra*I fragment. All lanes contain 3 μg of poly dI:dC and 5 μg of nuclear extract. The nuclear extracts were isolated from either S-phase-blocked cells (lane 2); cells released from the block for 3 h (lane 3); or cells released from the block for 6 h (lane 4).

with nuclear proteins from HeLa cell nuclear extracts. The sequence specificity of the bound complexes was assessed through competitive assays in which the nuclear extracts were incubated with the labelled *Hin*dIII–*Xho*I fragment as the probe, and increasing amounts of unlabelled *Hin*dIII–*Xho*I fragment were added as the competitor. If the binding of nuclear factors is specific, the unlabelled fragment should rapidly compete for the same factor binding to the labelled fragment and reduce the amount of labelled fragment bound in nucleoprotein complexes. The results shown in Fig. 4 (lanes 3–5) confirm the sequence specificity of the binding. As further confirmation of this, it was shown that a similar-sized unlabelled DNA fragment would not compete with probe for factor binding (Fig. 4, lanes 6 and 7).

(v) *Binding of nuclear proteins is dramatically reduced in DNA-synthesis-arrested cells*

Since the transcriptional block is abolished in HeLa cells treated with hydroxyurea and in cells arrested at the initiation of S phase, we wanted to determine whether there was a correlation between transcription readthrough and the factor binding described above. To analyse this possible correlation, HeLa cells were treated with 5 mM hydroxyurea for 5 h to inhibit DNA synthesis. Nuclear extracts from treated and untreated cells were isolated as described above. EMSAs were performed on these extracts in order to detect specific protein binding to the *Hin*dIII–*Xho*I fragment (Fig. 5). The results of these experiments indicate that there is a dramatic reduction in the amount of bindable protein that is present in the hydroxyurea-treated nuclei compared with the controls. The reduction in complex formation correlates with the loss of the block in transcription elongation seen in the hydroxyurea–treated nuclear run-on assays (Table 2).

As an extension of the binding data presented above using the 299 nt *Hin*dIII–*Xho*I fragment, subfragments within this region were assayed for binding activity. An 88 bp *Hin*dIII–*Dra*I fragment spanning the first *in vitro* transcription termination site (Fig. 1) was shown to bind nuclear factors. HeLa cells were synchronized and blocked at the initiation of S phase by sequential thymidine and aphidicolin treatment. Nuclear extracts were isolated from these S-phase blocked cells and from cells released from blockage and allowed to progress through S phase. EMSAs were performed on these extracts using the 88 bp *Hin*dIII–*Dra*I fragment. Results using this smaller fragment demonstrate that nuclear extract from S-phase-blocked cells forms one detectable fast migrating complex (Fig. 6, lane 2). However, upon release from the thymidine/amphicolin block, two additional major binding complexes are observed

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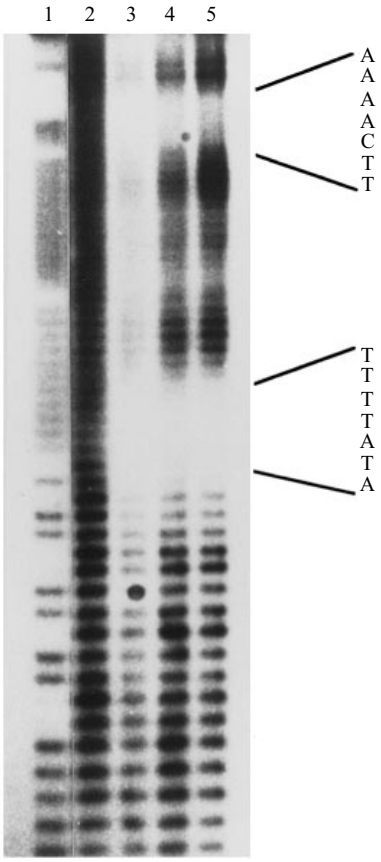

Fig. 7. Localization of DNA–protein binding using a MPE protection assay. Nuclear extract from untreated HeLa cells was incubated with labelled 88 bp *Hin*dIII–*Dra*I fragment followed by MPE footprinting as described in Sect. 2. Lane 1, Maxam/Gilbert sequencing reaction of the *Hin*dIII–*Dra*I fragment labelled at the *Hin*dIII site showing only pyrimidines. Lanes 2–5 contain the labelled HindIII–DraI fragment, 6 μg of poly dI:dC and 0·5 mM MPE. Lanes 3–5 contain decreasing amounts of HeLa nuclear extract (16, 8, 4 μg, respectively). The sequence reads: 5′ end at bottom of gel and 3′ end at top of gel.

(lanes 3 and 4, indicated by arrows). The appearance of these binding complexes correlates with the re-establishment of the block to transcription elongation (Table 2). Similar gel shift assays on other subregions of the 299 bp *Hin*dIII–*Xho*I fragment did not show any S-phase-specific shifts (data not shown).

(vi) *MPE footprinting of protein binding sites*

The location of protein–DNA interactions within the 88 bp *Hin*dIII–*Dra*I fragment was investigated by MPE footprinting. MPE induces random breaks in double-stranded helical DNA in the presence of ferrous ions and oxygen except where proteins are bound (see Section 2). Nuclear extracts from untreated HeLa cells were incubated with the labelled *Hin*dIII–*Dra*I fragment followed by MPE footprinting. As demonstrated in Fig. 7, two strongly protected regions are observed. The strongest protected region coincides with the 3′ end of the conserved region and overlaps with one of the three *in-vitro*-mapped termination sites previously described (Reines *et al.*, 1987). This region is approximately 7 bp long and contains the sequence 5′ATATTTT3′. The other protected region located 21 bp downstream is approximately 7 bp long and contains the sequence 5′TTCAAAA3′.

4. Discussion

Using tailed genomic subclones of human H3.3 as template, Reines *et al.* (1987) were able to demonstrate that highly purified calf thymus RNA polymerase II elongation is rapidly terminated within one of three discrete thymidylate stretches in the non-transcribed strand of intron 1. The *in vitro* transcription system utilized in these studies was devoid of RNA nucleolytic processing activities found in cell or nuclear extracts. Because the proteins and factors normally found in the nuclei were absent in their *in vitro* system, Reines *et al.* concluded that the attenuation of polymerase II elongation in this assay is most likely due to intrinsic polymerase and/or sequence properties.

In this report, we have used intron probes from regions immediately upstream and immediately downstream of the *in vitro* termination site to analyse the transcription block under more natural conditions. We have demonstrated that the transcription termination seen *in vitro* is also observed in isolated nuclei as a block to transcription elongation. Approximately three to fourfold more RNA polymerase II activity was seen to occur upstream of the *in vitro* termination site than downstream in actively proliferating HeLa cells. This transcription block seems to be abolished when DNA synthesis is halted either by hydroxyurea treatment or by arresting cells at the initiation of S phase by sequential treatment with thymidine and aphidicolin. Interestingly, the three T-runs corresponding to the *in vitro* block sites are located immediately 3′ of a 73 bp intron sequence that is 90% conserved over 250 million years of evolution. In fact, the first T-run is entirely contained within the 3′ end of the conserved region. The extraordinary conservation and size of this region suggested a role in maintaining regulatory function.

EMSAs were employed to determine whether any HeLa nuclear factors interact specifically with sequences spanning the termination sites. We were able to demonstrate that nuclear factors bind to this region in a sequence-specific manner. Of particular interest is the correlation between the translational block and the presence of a sequence-specific DNA binding activity in the nuclear extracts. This could indicate that this protein–DNA interaction is a component of the elongation pause/termination mechanism. A region of specific protein binding has been demonstrated by MPE footprinting within an 88 bp fragment that includes most of the conserved region and the first two *in-vitro*-mapped termination sites. This would agree with the mobility shift

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experiments using this fragment (Fig. 6), which shows two prominent shifts. The exact nature of the binding complexes has not been determined. Reddy & Reddy (1989) demonstrated that differential binding of nuclear proteins to the first intron of the c-*myb* gene is correlated with mRNA levels observed in different cells types. Their data imply that protein binding in the region of premature transcription termination may be involved in regulating RNA polymerase II elongation.

Kane and her colleagues have recently published a series of papers describing premature transcription termination of the H3.3 gene. They present very convincing data that not only can the termination event occur *in vitro*, independent of nuclear extracts, but that it also can be abolished by the addition of SII transcription elongation factor (Reines *et al*., 1987, 1989; SivaRaman *et al*., 1990). In addition, they have shown that the intrinsic termination that occurs *in vitro* is associated with a DNA bend that is centred at the *Dra*I site (Fig. 1) (Kerppola & Kane, 1990). It is unclear how the DNA bend supports transcription termination. Kerrpola & Kane suggest that additional interactions, such as a run of T residues, may be required.

The data presented here demonstrate that the transcription elongation block also occurs *in vivo* and, although not necessarily conflicting, would suggest that the *in vivo* control of transcription termination may involve specific protein interactions within this region. Our data would suggest that protein binding correlates with premature transcription termination. It is unlcear whether our observed protein binding is a causative agent or a result of the termination event. If SII does play a role in allowing readthrough, then the protein binding events seen here could act by counteracting the role of SII in masking the intrinsic termination site. It should be pointed out, however, that the specific role of protein binding to this region of the DNA has not been shown to induce transcription termination. We would suggest a model whereby, in a purified *in vitro* system, the bent DNA acts to cause intrinsic termination in concert with surrounding sequences. *In vivo* the RNA polymerase II associates with SII to allow readthrough of this termination site in non-S-phase cells. During S phase, however, a specific nuclear protein(s) binds to the region of the termination and inhibits the anti-termination activity of SII.

The significance of intragenic transcriptional arrest for a histone gene is uncertain. It has been suggested for the *Drosophila* heat shock genes (Rassmussen & Lis, 1993, 1995) that the elongational pause may allow a rapid rate of induction upon activation of the stress response. Although a different mechanism may be employed by the H3.3 gene, due to a greater distance of the elongational block downstream from the transcription initiation site, a similar functional role may be provided. Perhaps such a mechanism permits the transcriptional machinery to constitutively engage H3.3 while at the same time preventing the accumulation of full-length transcripts until a crucial developmental stage or time within the cell cycle. This mode of regulation would allow efficient and rapid reactivation of the gene upon receiving the appropriate stimulus.

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