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Effect of transcription of yeast chromatin on DNA topology *in vivo*

David S.Pederson[1] and Randall H.Morse

Laboratory of Cellular and Developmental Biology, Bldg 6, Rm B1-26, National Institutes of Health, Bethesda, MD, 20892 and [1]Department of Microbiology and Molecular Genetics, Given Bldg, University of Vermont School of Medicine, Burlington, VT 05405, USA

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Coding regions of transcribed and non-transcribed genes typically differ in chromatin structure. However, it is not known what kind of alterations in nucleosome or chromatin structure these differences reflect. To determine whether changes in nucleosome topology accompany transcription, we introduced into yeast a multicopy plasmid bearing the gene coding for the heat shock protein HSP26. The plasmid-borne gene is assembled into chromatin, and is induced by heat shock in the same manner as the endogenous *HSP26* gene. A small change in linking number in the *HSP26* plasmid accompanies heat shock. This change is consistent with that previously reported for thermal untwisting of DNA in yeast chromatin, and is equivalent in magnitude to that observed in control plasmids which lack heat-shock response elements. These data indicate that no stable alteration in nucleosome topology accompanies transcription of the heat shock gene. Moreover, the kinetics of the observed changes in linking number indicate that topoisomerase relaxes the thermally induced torsional stress in 1–5 min. We conclude that if alterations in nucleosome topology accompany polymerase passage, recovery must take place within this time period.

Key words: transcription/chromatin/DNA topology/yeast/heat shock

Introduction

How RNA polymerase transcribes DNA which is packaged in nucleosomes in eukaryotes is a long-standing question. Steric considerations (Morse and Simpson, 1988) suggest that nucleosomes in the path of transcribing RNA polymerase must be at least transiently perturbed. The increased sensitivity of the coding regions of actively transcribed genes towards nucleases (reviewed in Pederson *et al.*, 1986a; Yaniv and Cereghini, 1986; Conconi *et al.*, 1989; Perez-Ortin *et al.*, 1989) may reflect perturbation of nucleosomes during transcription. However, such measurements, by themselves, do not shed much light on the state of nucleosomes during transcription *in vivo*.

Ideas regarding how RNA polymerase reads nucleosome DNA fall into two extreme classes of models (reviewed by Pederson and Simpson, 1988). In the first, nucleosomes unfold by disruption of histone:histone contacts, while contacts between histones and (at least the non-coding strand of) DNA are preserved (Weintraub *et al.*, 1976; Prior *et al.*, 1983; Chen and Allfrey, 1987). In the second model, histone:DNA contacts are disrupted and the nucleosome dissociates (Karpov *et al.*, 1984). Histone:histone contacts may be preserved, with histone octamers being transiently displaced to associate with neighboring nucleosomes (Stein *et al.*, 1985). Transcription may actually affect both histone:histone and histone:DNA contacts (Nacheva *et al.*, 1989).

Recently, Lorch *et al.* (1987) found that histones were displaced from short linear fragments of DNA following *in vitro* transcription through a single nucleosome by RNA polymerase II or SP6 RNA polymerase. This finding supports the second class of models. However, in similar experiments, Losa and Brown (1987) and Lorch *et al.* (1988) found a nucleosome still present on 5S ribosomal DNA following passage by SP6 RNA polymerase, a result which is equivocal in terms of distinguishing between the two models. The different results of *in vitro* transcription experiments may be due to intrinsic differences in the rate of nucleosome reformation among different templates. Additionally, if nucleosome disruption entails displacement of the histone octamer, the presence of neighboring nucleosomes may enhance the efficiency of nucleosome reformation. Thus, the fate of nucleosomes during transcription *in vivo* is still an open question.

Freeing DNA from the histone octamer of a nucleosome releases a single negative superhelical turn in yeast (Pederson *et al.*, 1986b) as well as in larger eukaryotes (Germond *et al.*, 1975; Keller, 1975; Simpson *et al.*, 1985). If nucleosome disruption or unfolding takes place on a closed circular plasmid, relaxation of the released supercoil by topoisomerase will change the linking number by +1, an increase which can be measured by gel electrophoresis (Keller and Wendel, 1974). On this basis, we have designed an experiment to determine whether nucleosomes undergo stable changes in topology during transcription *in vivo*, and if so, how long they take to recover their normal structure.

The vehicle we have chosen for this work is a yeast multicopy plasmid bearing the *HSP26* gene (Petko and Lindquist, 1986). The plasmid-borne genes can be studied in active and inactive states which differ in rates of transcription by at least 100-fold, can be rapidly shifted between the two states, and represent a sufficiently homogeneous population to allow us to relate DNA topology to transcription and chromatin structure. We also take advantage of the property of thermal untwisting of DNA in yeast chromatin (Saavedra and Huberman, 1986; Morse *et al.*, 1987) to assay the kinetics of topoisomerase action on chromatin templates *in vivo*.

Results

Experimental strategy

Figure 1 schematically depicts plasmid chromatin at different stages in a transcription cycle to illustrate the experimental

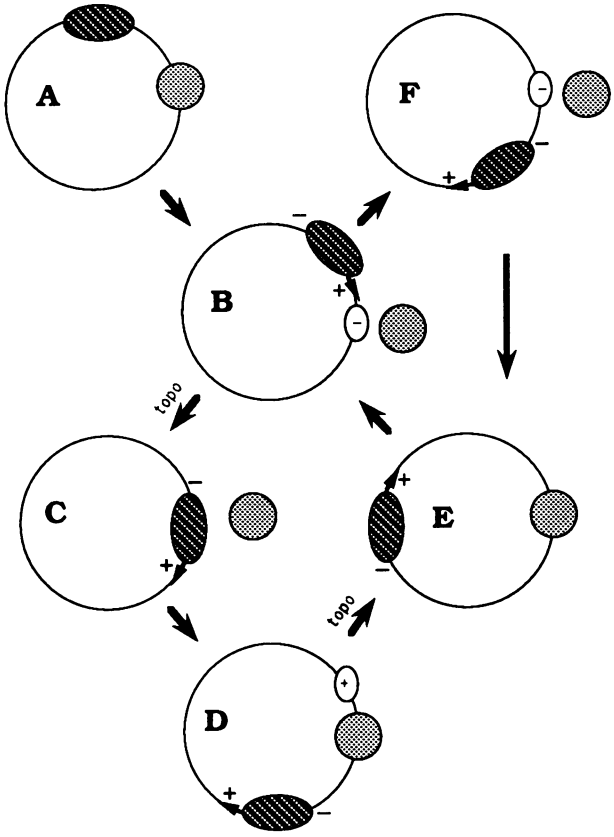

Fig. 1. Possible topological events during transcription of nucleosomal templates. To illustrate the experimental strategy used in this study, simple, circular templates are depicted, each containing one molecule of RNA polymerase (oval) and one nucleosome (circle). Transcription in B–F is clockwise. '−' and '+' symbolize negative and positive superhelical stress, respectively. In molecules B, C and F, nucleosome disruption is symbolized by displacement of the circle to the right of the template. This is not meant to imply a specific structure for the disrupted nucleosome. For further details, see the text.

strategy used in this study. 'A' represents a heat shock gene poised for induction. In yeast, the heat shock transcription factor is already bound to chromatin (Sorger *et al.*, 1987; Jakobsen and Pelham, 1988; D.Pederson, unpublished observations) and, for the present, we assume that RNA polymerase is bound to DNA, as it is in *Drosophila* (Gilmour and Lis, 1986). In 'B', transcription has been induced by heat shock. RNA polymerase, moving clockwise, generates positive superhelical density 3′, which is balanced by formation of negative superhelical density 5′ (Liu and Wang, 1987; Giaever and Wang, 1988; Wu *et al.*, 1988; Tsao *et al.*, 1989; Pruss and Drlica, 1989). One or more nucleosomes have been disrupted ahead of the polymerase molecule and, as a result, negative superhelical density is released. (The topological consequence of nucleosome unfolding is likely to be similar to that of nucleosome dissociation, although it may be somewhat less in magnitude, depending on the precise nature of the conformational change.)

In 'C', topoisomerase has acted to remove excess negative superhelical density. This changes the linking number by +1 per disrupted nucleosome (Keller, 1975; Germond *et al.*, 1975; Simpson *et al.*, 1985; Pederson *et al.*, 1986b). RNA polymerase transcribes the DNA which was associated with the disrupted nucleosome and, in 'D', the nucleosome reforms behind the polymerase. Note that 'C' and 'D' are

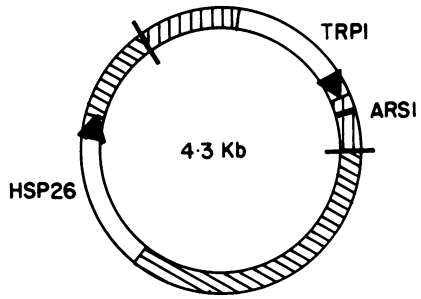

Fig. 2. The *HPS26* plasmid. Solid dark lines, perpendicular to the plasmid arc, indicate the junction between *TRP1*, *ARS1* and *HSP26* DNAs. Open regions indicate the *TRP1* and *HSP26* coding regions. Arrowheads indicate the direction of transcription.

topologically equivalent. In 'E', the positive superhelical density generated by nucleosome reformation has been removed by topoisomerase. The cycle can now repeat for passage through the next nucleosome.

In this study, we have tried to detect the topological intermediates represented by 'C' and 'D'. Detection of these intermediates depends on the rate of nucleosome disruption and reformation relative to the rate of topoisomerase action. At one extreme, nucleosomes over the entire coding sequence are disrupted for as long as transcription continues, and the intermediates would be readily detected by the procedures described below. At the opposite extreme, RNA polymerase transit and nucleosome reformation occur in a shorter time frame than the time it takes topoisomerase to act, a case which is illustrated by the cycle from 'B' to 'F' to 'E'. In this case, the topological intermediates 'C' and 'D' would not be formed. As is shown below, we have been able to measure the rate of topoisomerase action *in vivo* and, consequently, can set limits on the lifetime of intermediates 'C' and 'D'.

Transcription of HSP26 plasmid chromatin

Construction and copy number determination of the *HSP26* plasmid (Figure 2) are described in Materials and methods. In order to relate transcription to chromatin structure and nucleosome topology, it was first necessary to determine whether the multicopy plasmid behaves as a homogeneous population with respect to these properties. Lack of homogeneity would reduce the sensitivity of linking number determinations. Thus, we first asked whether the rate of transcription per molecule of *HSP26* plasmid is the same as it is for the chromosomal *HSP26* gene. To minimize the influence of the isolation procedure on the measured properties of the purified components (here, mRNA; later, DNA topoisomers), it was important to use as rapid a purification procedure as possible. Therefore, spheroplasts were made from log phase yeast cells with zymolyase, and allowed to recover metabolic activity in growth media supplemented with sorbitol for osmotic stability. This allowed rapid lysis of cells and simultaneous inactivation of all enzymatic activity by addition of a hot solution containing guanidium hydrochloride and sarkosyl (Holm *et al.*, 1986) to the spheroplasts in media. Typically, a slight increase in *HSP26* RNA abundance, and a decrease in abundance of TRP1 RNA, was observed following zymolyase treatment. However, during the recovery period, the abundance of several mRNAs, including *HSP26* RNA, returns to levels

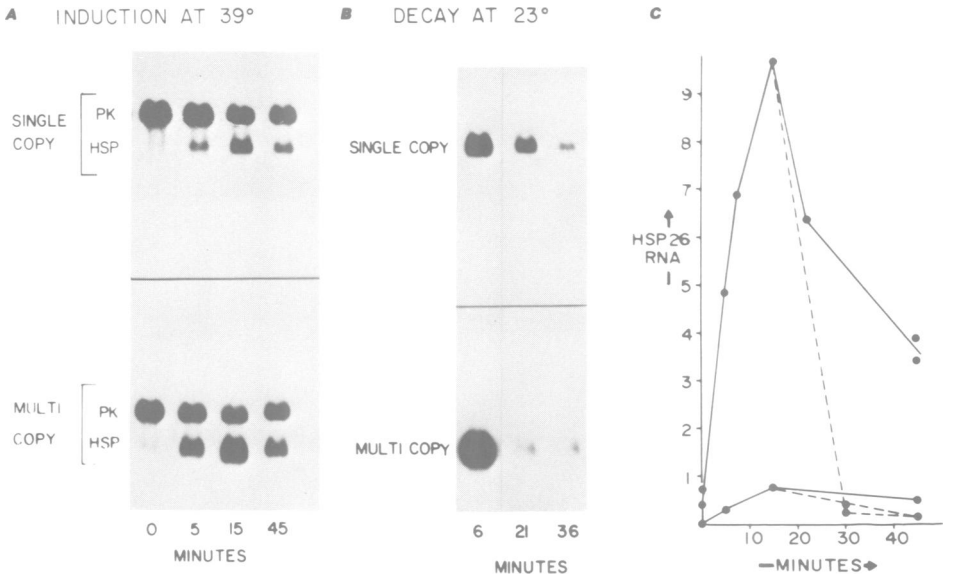

Fig. 3. *HSP26* RNA abundance before, during and after heat shock. (**A**) RNA was prepared from cells which lack ('single copy') and cells which contain ('multicopy') the *HSP25* plasmid, before and at varying times after induction of heat shock. Equal amounts of different RNA samples were electrophoresed, and visualized by hybridization with labeled *HSP26* and pyruvate kinase DNAs. (**B**) *HSP26* RNA in cells which had been heat shocked for 15 min, and returned to room temperature for varying lengths of time, was visualized as in panel (A). (**C**) *HSP26* message accumulation following heat shock. Values were determined from densitometric scans of autoradiograms of Northern blots, such as those in panels A and B. X-ray films were exposed for varying lengths of time to ensure that bands would be in the linear response range of the film. Preparation of RNA from heat shocked cells in these experiments entailed cells being at room temperature for about 6 min prior to lysis. The values plotted here have been corrected for this 6 min lag, assuming a 3 min half-life for *HSP26* RNA in cells containing the *HSP26* plasmid, a 15 min half-life for *HSP26* RNA in cells lacking the plasmid, and a simple exponential decay for each case. The values therefore represent calculated levels of accumulated RNA at the indicated times. Solid lines connect values obtained for varying lengths of heat shock, from two independent experiments. Dashed lines connect values obtained from cells after removal from heat shock. Upper solid line: *HSP26* plasmid-bearing cells; lower solid line: *HSP26* plasmid-free cells.

seen in intact cells (data not shown), indicating that zymolyase treatment does not cause prolonged transcription of the *HSP26* gene.

To compare transcription of the *HSP26* gene in cells containing or lacking the *HSP26* plasmid, cells of each kind were grown, spheroplasted and allowed to recover, all at room temperature. RNA was then purified from spheroplasts before or after rapidly shifting the temperature to 39°C by addition of hot medium, and again after returning heat shocked spheroplasts to room temperature. *HSP26* RNA was analyzed by Northern blot hybridization. In addition to loading gel lanes with equal amounts of RNA, hybridization to pyruvate kinase RNA was used to test for equal loading and possible RNA degradation. Figure 3A shows that, prior to heat shock, very little *HSP26* RNA is evident in either cells containing or lacking the *HSP26* plasmid. This suggests that if a transcriptional repressor is involved in *HSP26* gene regulation, it is not titrated out by the *HSP26* plasmid, which is present at roughly 95 copies per haploid genome. Upon heat shock, cells containing the *HPS26* plasmid accumulate more *HSP26* RNA per unit time than do parental cells, with accumulated message peaking about 15 min after initiation of heat shock in either case.

The difference in *HSP26* RNA accumulation between heat shocked cells having or lacking the *HSP26* plasmid is only 10- to 15-fold (Figure 3A,C), well below the nearly 100-fold difference in gene copy number. However, the amount of message accumulated is a function both of mRNA production and decay. We therefore measured decay rates for the *HSP26* message in both cell types. Cells were heat shocked for 15 min, then returned to room temperature for varying times before isolation of RNA. Figure 3B shows that *HSP26* RNA decays considerably faster in cells containing the *HSP26* plasmid than in cells which lack the plasmid. Fitting these and similar data to simple exponential decays indicates that the *HSP26* message has a half-life of about 15 min in cells lacking the *HSP26* plasmid, and 3 min in cells containing the plasmid. This 5-fold increase in decay rate, together with the 10- to 15-fold increase in abundance, suggests that each copy of the *HSP26* plasmid has roughly the same transcriptional activity as does the endogenous gene.

Our ability to detect topological intermediates may depend on the absolute rate of transcription of the *HSP26* gene. For example, if transcription rates are extremely low, only a minority of templates will be perturbed at any given time, and detection of intermediates will be difficult. Thus, *HSP26* RNA amounts per cell were quantified by comparison to known amounts of *in vitro* synthesized RNA (see Materials and methods). At least 1−2 transcripts per gene per min accumulate during 15 min of heat shock. Given a mRNA half-life of 3 min, this corresponds to an average transcription rate of 4 transcripts per gene per min. Thus, unless the nucleosome disruption/reformation cycle (Figure 1) occurs within 15 s, we expect that *HPS26* chromatin will be in a continuously disrupted state during transcription.

Plasmid and genomic HSP26 chromatin structures are indistinguishable

To determine whether the *HSP26* plasmid is homogeneously packaged in chromatin, plasmid chromatin was compared with genomic *HSP26* chromatin by nuclease cleavage and indirect end-labeling (Nedospasov and Georgiev, 1980; Wu, 1980). If a significant fraction of plasmid molecules were transcriptionally inactive, while another fraction was active,

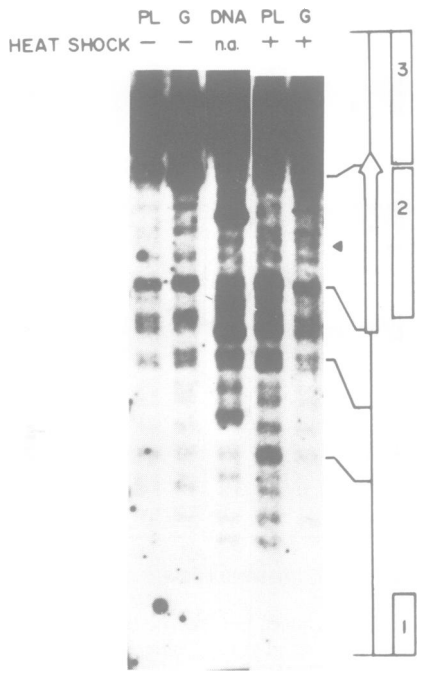

Fig. 4. Indirect end-label analysis of micrococcal nuclease cuts in the *HSP26* gene. Purified *HSP26* plasmid DNA ('DNA'), nuclei from non-transformed control and heat shocked cells ('G'), and nuclei from *HSP26* plasmid-containing control and heat shocked cells ('PL') were treated with micrococcal nuclease, and DNA purified. DNA was cut with *Bam*HI and *Pst*I, electrophoresed, blotted to nitrocellulose and hybridized with a probe (*Bam*HI−*Hin*cII) which is designated '1' in the drawing of the *HSP26* transcription unit at the side. The filled arrowhead indicates a cut which is more prominent in chromatin from heat shocked cells and in purified DNA, than in non-heat shocked chromatin. Subsequently, blots were reprobed with probe '3' (*Nru*I−*Pst*I; data not shown). Probe '2' (*Bgl*II−*Nru*I) was used in the experiment shown in Figure 5.

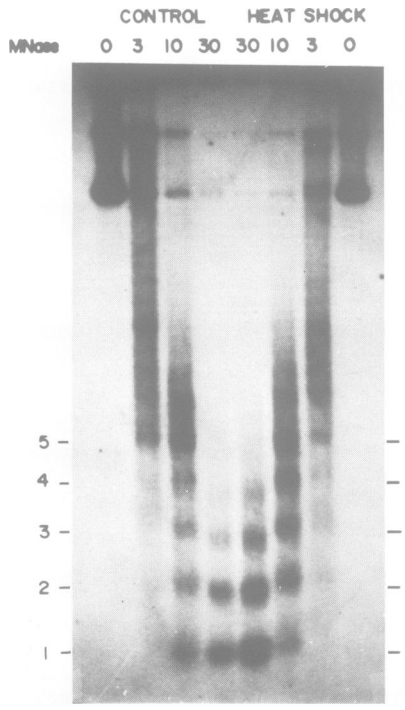

Fig. 5. HSP26 DNA is packaged in nucleosomes. Nuclei from control and heat shocked cells containing *HSP26* plasmid were treated with 30, 100 or 300 unit × min × ml^{-1} micrococcal nuclease. DNA was purified, electrophoresed, blotted to nitrocellulose and hybridized with probe '2' shown in Figure 4. The unit size oligomer is about 160 bp.

the pattern or intensity of nuclease cleavage sites might be altered in the plasmid genes, relative to the chromosomal gene. Nuclei from cells containing or lacking the *HSP26* plasmid were treated with micrococcal nuclease (MNase). DNA was purified and nuclease cuts mapped using probes derived from both 5′ and 3′ of the *HSP26* gene. As can be seen in Figure 4, the pattern and intensity of cuts on genomic and plasmid substrates are virtually identical, indicating that most or all of the plasmid molecules are packaged in a manner identical to that of the endogenous gene. Similar results were obtained wtih DNase I (results not shown).

While a detailed analysis of *HSP26* chromatin structure will be presented elsewhere, comparison of cleavage patterns of chromatin and naked DNA suggests that the transcribed region of the *HSP26* gene is associated with nucleosomes. To confirm this inference, DNA from nuclei treated with micrococcal nuclease was electrophoresed, blotted to nitrocellulose, and hybridized with a probe from the *HSP26* coding region (probe number 2 in Figure 4). As can be seen in Figure 5, *HSP26* DNA is cleaved by micrococcal nuclease into an oligomeric series of DNA fragments that is characteristic of DNA packaged in a nucleosomal array.

Effect of transcription on HSP2 chromatin structure and nucleosome topology

The pattern and intensity of MNase (Figure 4) and DNase I (not shown) cuts in *HSP26* chromatin are largely unaltered following induction of transcription by heat shock. However, weak cuts which occur in the coding region of naked DNA, but are suppressed in non-heat shocked chromatin, are found in heat shocked chromatin (e.g., filled arrowhead in Figure 4). This is consistent with alterations in nucleosome structure, position, or stability.

The absence of more dramatic changes in the coding region of *HSP26* chromatin following induction of transcription raised the possibility that *HSP26* chromatin exists in a 'transcriptionally competent' state prior to heat shock. If this were the case, deletion of promoter sequences 5′ to the *HSP26* gene might alter the chromatin structure over the gene's coding region. To test this possibility, a 1.5 kb restriction fragment immediately 5′ to the *HSP26* coding region was removed from *HSP26* plasmid, and the resulting plasmid introduced into yeast. The chromatin structure of the *HSP26* coding region sequences in this plasmid was identical to that of the inactive endogenous and plasmid *HSP26* genes, as assayed by MNase and restriction endonuclease digestion (data not shown).

To determine whether nucleosome unfolding or disruption accompanies transcription of the *HSP26* gene, we examined the topology of *HPS26* plasmid DNA before, during and after heat shock. As discussed earlier, loss or unfolding of nucleosomes, followed by topoisomerase action, would result in an increase in the linking number. Increasing the temperature has an opposite effect of smaller magnitude, due to thermal untwisting of the DNA (Saavedra and Huberman, 1986; Morse *et al.*, 1987). For the 16°C rise in temperature used in this experiment, thermal untwisting of the DNA in yeast chromatin results in a change in linking number of about −0.35 per kb, or about −1.5 for the *HSP26* plasmid. This latter effect, specific to yeast so far as is presently

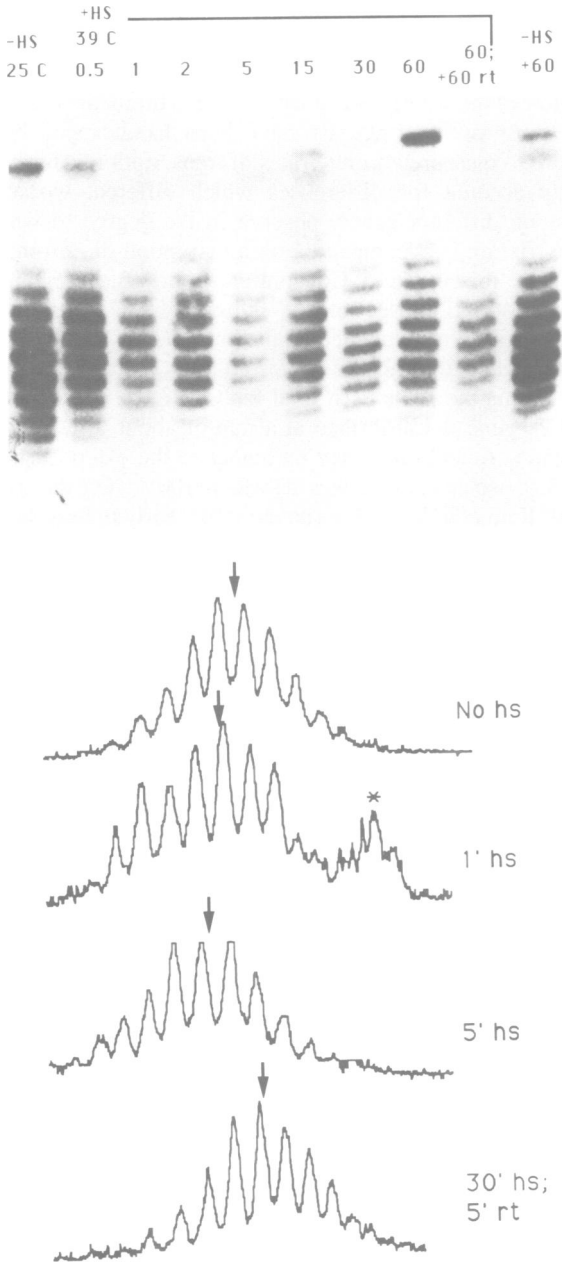

Fig. 6. Topology of the *HSP26* plasmid before, during and after heat-shock. **Upper panel**: spheroplasts were prepared from cells carrying the *HSP26* plasmid and allowed to recover at room temperature for 1 h. An aliquot of these cells was rapidly lysed and DNA isolated (−HS, 25°C). Another aliquot was reserved as a non-heat shock control; DNA was isolated after 60 min further incubation (−HS; +60). The remaining culture was subjected to rapid heat-shock and maintained at 39°C, and aliquots lysed at various times, as indicated. Finally, an aliquot of the heat-shocked spheroplasts was allowed to recover at room temperature for 1 h, then lysed (+HS 60; +60 rt). The bands at the top are due to nicked circular *HSP26*. **Lower panel**: densitometric scans of topoisomer distributions from an independent experiment in which spheroplasts were treated as above. The arrows indicate the measured centers of the topoisomer distributions; more negatively supercoiled topoisomers are at the left. The peak labeled with an asterisk in the second trace is due to a smudge on the autoradiogram.

known (Morse and Cantor, 1985; Ambrose *et al.*, 1987), allows the following important control experiment to be done. Plasmids lacking any heat shock inducible promoter can be isolated from cells before, and at varying times after,

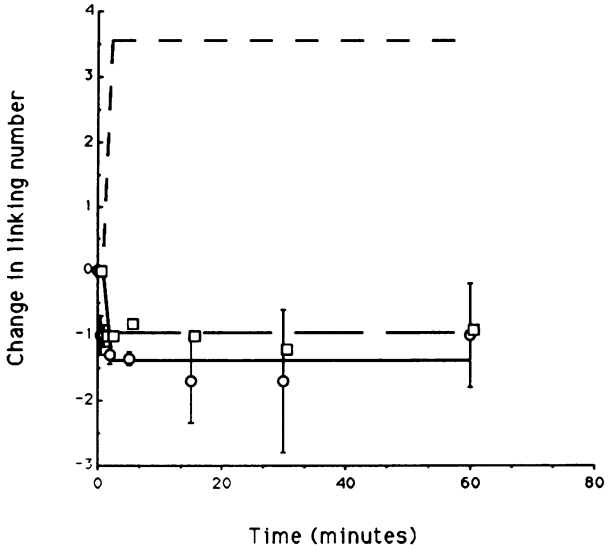

Fig. 7. Change in linking number of the *HSP26* plasmid after heat shock. The linking number of the *HSP26* plasmid (circles) after heat shock relative to the linking number prior to heat shock was determined from data such as that of Figure 6. Data points and standard deviations derive from four topoisomer gels covering three independent experiments. The solid line shows the linking number change expected if the only change is due to thermal untwisting of the plasmid chromatin, and takes 5 min to be completed. The dashed line shows the change expected if, in addition to the change due to thermal untwisting, five nucleosomes on each plasmid were displaced or completely unfolded for the duration of heat shock. The squares show the measured change in linking number following heat shock for the 2.8 kb GAT2 plasmid, and the long dashes indicate the expected change due to thermal untwisting.

shifting the temperature from 23 to 39°C, and at varying times after returning cells to 23°C. By following the kinetics of the thermally induced change in linking number, one can determine how quickly topoisomerase responds to torsional stress in the plasmid chromatin template. When the kinetics of relaxation of torsional stress induced by thermal untwisting were followed in control plasmids (lacking heat shock inducible elements) by shifting the temperature from 20−25°C to 39°C, a rapid re-equilibration of linking number was observed. Some change in linking number was obvious after 30 s, the shortest time measured, and relaxation to the new linking number was complete in 1−5 min (data not shown, but see Figure 7). This indicates that under the conditions of our experiment, we could expect to detect changes in nucleosome topology persisting for longer than 1−5 min, and possibly for even shorter duration.

Similar results were obtained from measurements on cells containing the *HSP26* plasmid, as shown in Figure 6. After a slight shift in the topoisomer distribution to more negative supercoils (upward in the chloroquine-containing gel of the top panel), complete in about 1 min, no further change takes place. Precise measurement of the centers of the topoisomer distributions from three independent experiments are summarized in Figure 7. Apart from a rapid (1−5 min) shift in response to thermal untwisting, no change in linking number takes place. In particular, no shift to more positive supercoiling (dotted line in Figure 7), as would result from nucleosome dissociation or unfolding, is seen. Returning heat shocked cells to room temperature by addition of chilled media results in recovery of the original topoisomer distribution within 5 min (Figure 6B, last trace, and data not shown).

To examine the possibility that use of spheroplasts affected our results, plasmid DNA was isolated from intact cells, using a glass bead lysis procedure (Saavedra and Huberman, 1986), and its topology compared with plasmid DNA from spheroplasts. Relative to intact cells, the *HSP26* plasmid linking number was slightly greater immediately after zymolyase treatment (+0.7), and after metabolic recovery (+0.5). However, comparable shifts were seen in the same experiment for a control plasmid lacking heat-shock-inducible elements, and are therefore unlikely to be related to changes in transcription.

Discussion

We have found that the *HSP26* gene, when present in a multicopy plasmid, is transcriptionally induced by heat shock to levels comparable on a per copy basis to that of a endogenous *HSP26* gene. Transcriptional induction is accompanied by only slight alterations in chromatin structure, as assessed by cleavage with MNase and DNase I. No change in DNA topology, as would be expected to accompany stable nucleosome unfolding or disruption, was observed.

Chromatin structure of the HSP26 gene before and after heat shock

The *HSP26* gene appears to be packaged into nucleosomes both before and after heat shock (Figures 4 and 5). It is possible that episomal chromatin does not faithfully mimic some features of genomic chromatin, such as higher order structure and involvement with the nuclear matrix. However, in the assays presented here, plasmid and genomic copies of the *HSP26* gene appear to be identical in chromatin structure, consistent with other studies which compared plasmid and genomic chromatin (Bloom and Carbon, 1982; Perez-Ortin *et al.*, 1987). In the case of the yeast *TRP1* gene, differences in chromatin structure between plasmid (Thoma *et al.*, 1984) and genomic (Lohr and Torchia, 1988) copies may be due to the fact that construction of the TRP1ARS1 plasmid created a junction sequence only 103 bp from the *TRP1* open reading frame.

Our finding that heat shock does not substantially alter MNase or DNase I sensitivity or pattern of cleavage of the *HSP26* gene is consistent with other studies showing that transcribed genes may still be packaged into nucleosomes (e.g. Lacy and Axel, 1975; Giri and Gorovsky, 1980; Bloom and Anderson, 1982; Benezra *et al.*, 1986; DeBernardin *et al.*, 1986; Richard-Foy and Hager, 1987; Studitsky *et al.*, 1988; Ip *et al.*, 1989). In other cases, however, transcribed genes have been found to undergo loss of nucleosomes or alterations in chromatin structure interpreted as an unfolding or disruption of normal chromatin structure (reviewed in Pederson *et al.*, 1986a; Yaniv and Cereghini, 1986; Bjorkroth *et al.*, 1988; Conconi *et al.*, 1989). In some cases it is clear that the alteration in chromatin structure is not directly coupled to transcription (Bloom and Anderson, 1982; Rose and Garrard, 1984; Ip *et al.*, 1989; Pavlovic *et al.*, 1989). In the case of the *HSP26* gene, examination of Figure 4 shows that nuclease cleavage occurs more frequently and with greater intensity in the 5′ region of the gene than in the coding region for both naked DNA and chromatin. Thus, the absence of intense cuts in the coding region of induced *HSP26* chromatin may simply reflect an intrinsic resistance of these sequences to cleavage by MNase and DNase I. Such heterogeneity in cuts made on naked DNA by MNase has been observed previously (e.g. Keene and Elgin, 1984).

Most of the studies which have related chromatin structure and transcriptional activity have been handicapped by a failure to measure absolute rates of transcription. This may partly account for differences which different workers, studying different genes, observe in the degree to which transcription is accompanied by a disruption of chromatin structure (discussed by Conconi *et al.*, 1989). To estimate the transcription rate of the induced *HSP26* gene, we measured the absolute accumulation of *HSP26* RNA after heat shock. If the half-life of *HSP26* RNA early in induction at 39°C is the same as it is at 23°C [Figure 3B,C], each *HSP26* gene is transcribed at a rate of about 4 transcripts per min. Actual rates may be higher to the extent that the yield of isolated RNA was less than 100%. On the other hand, if no *HSP26* RNA turnover occurs early in heat shock, this value may be as much as a 5-fold overestimate. However, fairly rapid turnover probably does occur at 39°C, in view of the decline in *HSP26* RNA abundance during a continuation of heat shock from 15 to 45 min (Figure 3A,C). We conclude that the HSP26 plasmid represents a good model for studying alterations in chromatin structure associated with a highly active gene.

Topoisomerase rapidly relaxes torsional stress in minichromosomes in yeast

Both *HSP26* and control plasmids exhibit a change in linking number in response to an increase in temperature. The onset of this change in linking number is visible as early as 30 s following the temperature shift, and the shift is complete within 1 to 5 min (Figures 6 and 7). Thus, topoisomerase responds to torsional stress in yeast in 1−5 min, or less. This is a maximum estimate since the kinetically slow step in our experiment could be the thermal untwisting of nucleosomal DNA, and not topoisomerase action. The measured change in linking number due to thermal untwisting shows that topoisomerase is responsive to even small amounts of torsional stress, and so would be expected to respond to the stress resulting from unfolding or disruption of nucleosomes.

The rapid relaxation of the small amount of torsional stress induced by thermal untwisting in yeast minichromosomes is consistent with generation of excess positive or negative supercoiling in transcribing yeast minichromosomes, under differing conditions of topoisomerase inactivation. In these experiments, excess positive supercoils (Giaever and Wang, 1988) or negative supercoils (Brill and Sternglanz, 1988) were attributed to relaxation of superhelical density on only one side of the RNA polymerase molecule. For this relaxation to take place, topoisomerase activity must be faster than the diffusional cancellation of oppositely handed supercoils. Such rapid relaxation seen in this and in the present work, would appear to be inconsistent with models invoking stable maintenance of torsional stress during transcriptional activation (Ryoji and Worcel, 1984; Villeponteau *et al.*, 1984). However, we cannot rule out the possibility that larger eukaryotes have means (e.g. linker associated H1- or H5-like histones which are probably absent in *Saccharomyces cerevisiae*) (Perez-Ortin *et al.*, 1989) for stabilizing torsional stress which are not available to yeast.

Nucleosome topology during HSP26 transcription in yeast

During transcription, several events could contribute to changes in DNA topology. Binding of inducer proteins could affect DNA topology by altering DNA twist or by causing DNA to bend, as is the case for the transcription factor TFIIIA (Reynolds and Gottesfeld, 1983; Bazett-Jones and Brown, 1989; Schroth *et al.*, 1989). However, in the cases documented to date, these effects appear to be small. Moreover, because yeast heat shock factor appears to be constitutively bound to its target sequences (Sorger *et al.*, 1987; Jakobsen and Pelham, 1988), including those in the *HSP26* promoter (D.Pederson, unpublished observations), its binding should not affect our experiments.

Association of one molecule of RNA polymerase II with DNA during transcription alters DNA topology by unwinding about 14 bp of the duplex (Pedone and Ballario, 1984). Such polymerase-induced unwinding was interpreted to be the cause of a transcriptionally induced decrease in linking number of a plasmid containing a 7 kb *CYC1-lacZ* fusion gene (Osborne and Guarante, 1988). The linking number change was stable, inasmuch as it was preserved during a DNA isolation which involved preparing spheroplasts in the absence of nutrients. In contrast, our experiments were designed to detect transient events, which may be superimposed on such stable changes as the association of RNA polymerase molecules with DNA. Assessing the influence of polymerase loading on our results requires estimating the number of RNA polymerase molecules which associate with the *HSP26* gene following induction of transcription. Extrapolation of Osborne and Guarante's (1988) results to a transcribed region of 750 bp (the length of the *HSP26* gene), would lead to a prediction of a linking number change of −0.6, near the limits of detection in our experiments. Our estimate of transcription rates for the *HSP26* gene corresponds to roughly 50 nucleotides of RNA synthesized per gene per second. This probably requires continuous activity by at least one molecule of RNA polymerase.

The binding of one or more additional molecules of RNA polymerase to DNA after induction of transcription would create torsional stress that is opposite in sign to the change associated with nucleosome unfolding or loss. If this occurred during induction of *HSP26* gene transcription, the negative supercoils generated by nucleosome disruption could be balanced by polymerase binding, leaving a net linking number change of zero. For this to occur, the two effects would have to be coincidentally equal in magnitude throughout the period of *HSP26* transcription. If, instead, molecules of RNA polymerase become fully engaged prior to maximal nucleosome disruption, we might expect to see a transient decrease in linking number, followed by an increase. This was not observed. Furthermore, when transcription was halted by removing cells from heat shock, the topoisomer distribution rapidly (<5 min) reverted to its original form (Figure 6B, last trace, and data not shown). This suggests that nucleosomes, if disrupted, must have reformed within 5 min following transcription cessation. The measured shifts in linking number could be reconciled with polymerase unwinding and/or nucleosome disruption, if both events were completed before topoisomerase responded to the transient torsional stress induced. But this would be consistent with our conclusion that no stable changes in nucleosome topology accompany transcription of the *HSP26* gene.

A third phenomenon related to DNA topology during transcription is segregation of positive and negative superhelical domains by transcribing polymerases, as described by Liu and Wang (1987). This is symbolized in Figure 1 by a '+' on the leading side of RNA polymerase, and a '−' on the trailing side. This phenomenon has been demonstrated *in vivo* only by experiments in which topoisomerase activity is altered, and would not be expected to contribute to a linking number change in our experiments. We note that positive superhelical stress ahead of the polymerase would be reduced by nucleosome disruption, and that negative superhelical stress behind the polymerase would be reduced by nucleosome refolding or reformation. Thus, these events may be temporally and energetically coupled. Positive superhelical density induced by elongating RNA polymerase may promote nucleosome disruption ahead of the polymerase, while negative superhelical density may promote reformation behind. If this occurs, a nucleosome disruption−reformation cycle might be complete within a few seconds, or the time it takes RNA polymerase to read a few hundred base pairs of DNA. This picture is consistent with the present results, and also with those of DeBernardin *et al.* (1986) and Bjorkroth *et al.* (1988), showing that nucleosomes appear to be present very close to transcribing RNA polymerase II in SV40 minichromosomes and in the Balbiani ring genes in the salivary glands of *Chironimus tentans*, respectively.

Materials and methods

Plasmid construction, transfection, and copy number

The yeast *HSP26* gene, cloned in pUC12 and designated 26PBm (Petko and Lindquist, 1986), was kindly supplied by Dr S.Lindquist. A *Hin*dIII−*Bam*HI fragment bearing the *HSP26* gene was cloned into the *Bam*HI site of pBRAT2, a TRP1ARS1-containing plasmid described earlier (Morse *et al.*, 1987), by standard techniques (Maniatis *et al.*, 1982). After amplification in *E.coli*, the plasmid was cut with *Eco*RI to separate yeast and *E.coli* sequences. The linear yeast fragment was circularized with ligase to form the *HSP26* plasmid depicted in Figure 2, and transfected into the haploid, *trp1*− yeast strain SC3 (Sigurdson *et al.*, 1981) by the method of Ito *et al.* (1983).

A promoterless version of the *HSP26* plasmid was constructed by deleting a 1.5 kb fragment encompassing sequences from the *Bgl*II site at −55 with respect to the *HSP26* start site (Bossier *et al.*, 1989), to the 5′ *Bam*HI site. These sequences are necessary for *HSP26* gene activity (Petko and Lindquist, 1986). The plasmid GAT2 (Morse *et al.*, 1987) was used as a control for measuring linking number shifts due to thermal untwisting. Because topoisomers from the 2.8 kb GAT2 plasmid can be electrophoretically separated from those of the 4.3 kb *HSP26* plasmid, direct comparison of the kinetics of linking number change between the two plasmids could be made in mixing experiments.

To determine plasmid copy number, total DNA from plasmid bearing cells was cut with *Bam*HI and *Eco*RI. This allows electrophoretic separation of plasmid and endogenous fragments containing the *HSP26* gene. These fragments were visualized by Southern blotting and quantified by scintillation counting. Given that the endogenous *HSP26* gene is single copy (Petko and Lindquist, 1986), we calculate a plasmid copy number of about 95 per haploid genome.

Cell growth, spheroplasting and heat shock

Yeast was grown in 2% dextrose, 0.67% nitrogen base without amino acids, 25−50 mM potassium phthallate (pH 5.5 with NH_4OH) and 0.002% each uracil and histidine to a density of $0.67-2 \times 10^7$ cells per ml. Cells for control experiments involving plasmids lacking heat shock inducible elements were grown at 30°C; otherwise growth was at room temperature (20−25°C). Spheroplasts were made as described (Pederson *et al.*, 1986b), except that all operations were carried out at room temperature. Spheroplasts were

collected and suspended at a concentration of $2.7-8 \times 10^7$ cells/ml in growth media lacking phthallate and supplemented with 1 M sorbitol. They were then allowed to recover metabolic activity while gently shaking (60 r.p.m.) at room temperature for 1 h. Heat-shock was initiated by addition of an equal volume of media at a temperature calculated to bring the culture to 39°C, and the culture immediately placed into a water bath at 39°C. Cells were removed from heat shock by allowing cultures to cool in air or, in one experiment, by addition of chilled medium.

RNA and DNA isolation and analysis

Nucleic acids were isolated from cultures before, during, or after heat shock by addition of an equal volume of hot (65°C) buffer containing Sarkosyl and guanidinium–HCl (Holm *et al.*, 1986). After ethanol precipitation and (for DNA) treatment with RNase A, nucleic acids were treated with 0.5% SDS and 0.5 mg/ml proteinase K, extracted with phenol and chloroform, and reprecipitated.

RNA from 10^7-10^8 cells was electrophoresed on 5% polyacrylamide gels containing 7 M urea or on agarose gels containing either methylmercury or formaldehyde (Maniatis *et al.*, 1982). Acrylamide gels were electroblotted to nylon membranes at 18–20 V (400 mA) for 2–3 h with an electrophoretic transfer system (E-C Electroblot) in 0.3X Tris-acetate-EDTA gel buffer. Blotting of agarose gels to nylon or nitrocellulose filters, and hybridization of filters, was as previously described (Pederson *et al.*, 1984; Morse *et al.*, 1987). Probes for *HSP26* and pyruvate kinase (kindly provided by Dr A.Hinnebusch) were gel purified and labeled by nick translation.

DNA topoisomers were separated by electrophoresis on 1.4–1.5% agarose gels containing 40 μg/ml chloroquine phosphate (Sigma) at 2 V/cm for 22–26 h, and visualized by blotting to nylon membranes and hybridization, as described previously (Morse *et al.*, 1987). In addition to determining the centers of the topoisomer distributions as described previously (Morse *et al.*, 1987), we also attempted to fit the observed topoisomer intensities as Gaussian distributions on log plots (Depew and Wang, 1975). A poor fit might arise from structural or functional heterogeneity in the plasmid chromatin population. In most cases, the fit was extremely good ($r^2 > 0.97$) In all cases in which the Gaussian and weight-average centers could be compared, they agreed within linking numbers of 0.1.

HSP26 RNA quantitation

To quantify *HSP26* mRNA, a *Bgl*II–*Nru*I fragment from −55 to +687 relative to the *HSP26* start site (Bossier *et al.*, 1989) was cloned in the sense orientation next to the T7 promoter of a vector derived from pGEM4 (Promega Biotech) to yield the plasmid pGEMHSP. After cleavage with *Ava*II, transcription of pGEMHSP by T7 RNA polymerase yields a run-off transcript of about 420 nt, 40 nt of which are derived from the vector and the rest from the HSP26 gene. The run-off transcript was quantified by dividing a transcription reaction into two equal portions and supplementing one portion with a small amount of radiolabeled CTP. Labeled transcripts were separated by gel electrophoresis, and the band corresponding to the *Ava*II run-off transcript was excised and quantified by scintillation counting. This provided a calibration of the specific transcript in the unlabeled reaction; serial dilutions of the unlabeled RNA were then electrophoresed along with total message from a known number of yeast cells ($0.5-1 \times 10^7$ cells), blotted and hybridized with nick-translated pGEMHSP. Densitometry of the resulting autoradiograms was used to calculate the absolute amount of HSP26 message per cell. No corrections were made for possible non-quantitative recovery of RNA from cells, or for the small difference in target size between the *Ava*II run-off transcript and the *HSP26* message.

Analysis of chromatin structure

Before or following heat shock of metabolically recovered spheroplasts, phenylmethylsulfonyl fluoride, Pepstatin A, and iodoacetate were added to cultures to a final concentration of 0.5 mM, 2 μg/ml, and 1 mM, respectively. (These inhibitors were also freshly added to all buffers used in isolating nuclei, but omitted during treatment of nuclei with nucleases.) Subsequent operations were at 0–4°C. About 6×10^9 spheroplasts were collected in a JA10 rotor, 5 min, 4000 r.p.m., and suspended in 6 ml buffer A (80 mM KCl, 5 mM $MgCl_2$, 10 mM MOPS, pH 7.3 with NaOH, 1 mM EGTA, 0.5 mM spermidine–HCl) containing 18% Ficoll. Spheroplasts were allowed to swell for 10–15 min and lysed with a Teflon homogenizer. Crude nuclei were collected in a JA20 rotor, 5 min, 13 000 r.p.m., and suspended in 6 ml buffer A. Nuclei were collected in a JA20 rotor, 5 min, 6500 r.p.m., washed once with 6 ml buffer B (200 mM NaCl, 5 mM $MgCl_2$, 10 mM PIPES, pH 7.3 with NaOH, 0.5 mM EGTA, 0.1% β-mercaptoethanol) and suspended in 1 ml buffer B.

Suspensions of nuclei were mixed with 1/4 volume of buffer C (25 mM HEPES, pH 7.6 with NaOH, 12.5 mM $MgCl_2$, 12.5 mM $CaCl_2$), containing appropriate amounts of MNase or DNase I, and digested at 37°C for 2 min. Digestions were stopped by addition of 1/3 volume of buffer D (50 mM EDTA, pH 7.6 with NaOH, 1% SDS, 0.4 mg/ml proteinase K). After treatment at 50°C for >60 min, SDS:protein complexes were precipitated by addition of potassium acetate. Nucleic acids were precipitated with ethanol, and DNA further purified by treatment with RNase A, extraction with phenol and chloroform, and reprecipitation. Cleavage sites were mapped by the method of indirect end labeling (Wu, 1980; Nedospasov and Georgiev, 1980), using an upstream fragment (*Bam*HI–*Hin*cII; probe 1, Figure 4) or downstream fragment (*Pst*I–*Nru*I; probe 3, Figure 4) as probe.

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