Transcription of eucaryotic tRNA^{met} and 5SRNA genes by RNA polymerase III is blocked by base mismatches in the intragenic control regions

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ABSTRACT

We have constructed duplex DNAs containing single G-T or A-C mismatches in the *X. laevis* tRNA^{met} gene. Mismatches within regions of this gene which are bound by transcription factor TFIIC prevent transcription by RNA polymerase III. Homoduplexes with G-C-A-T mutations at some of the same sites, however, are transcribed efficiently in oocytes. Mismatches outside of the tRNA^{met} gene have no effect upon transcription. A survey of several point mutants in the Syrian hamster 5SRNA gene indicates that mismatches outside the internal control region somewhat reduce transcription, but a mismatch within the internal control region blocks transcription. Thus, the presence of mismatched bases in the region of DNA which interacts with RNA polymerase III transcription factors blocks transcription, perhaps by interfering with DNA renaturation following transit of the RNA polymerase.

INTRODUCTION

The transcriptional competence of tRNA genes, 5SRNA and adenovirus VA RNA genes is determined by their association with several transcription factors, in addition to RNA polymerase III (1,2). These proteins assist in the binding of RNA polymerase (2-5). They may also determine whether the DNA is organized into transcriptionally active chromatin (6-8), and they may participate directly in transcription by interacting with the template or with RNA polymerase during catalysis.

Genes encoding 5SRNAs contain an internal control region of approximately 40 bp, much of which interacts with transcription factor TFIIA (9-13). Sequences near the 5' and 3' ends of the internal control region are important for the action of additional transcription factors TFIIB and TFIIC (14-17). Although the order of binding of these factors to the template DNA has been established (2-5), their structural relationship to each other, to the template, and to RNA polymerase III in a stable transcription complex is not yet clear.

Transcription of tRNA and adenovirus VA genes does not require TFIIA (1-2). Binding of TFIIC protein(s) occurs to internal sequences near the 3' end of these genes and, depending upon the gene, may extend to the 5' end (5,18-21). Analyses of the transcription of mutationally altered tRNA genes have uniformly agreed upon the importance of sequences near the 3' end of the gene (the "B block"), but have differed about the importance of sequences

upstream of this element (22-31). This lack of agreement is perhaps not surprising; tRNA genes differ significantly in sequence and a single transcription factor may form multiple contacts in binding. Alternatively, there is no compelling reason to suspect that a single transcription factor is responsible for the activation of all tRNA genes and of other genes such as the VA RNA genes. Several related factors with overlapping specificities may serve this function.

Our previous experiments with the *X. laevis* tRNA₁^{met} gene identified intragenic G-C base pairs which were important for expression in oocytes. We have further analyzed the importance of these sites by forming heteroduplexes containing single base mismatches and by measuring their transcription in oocytes. We find that DNAs containing mismatched bases within the tRNA₁^{met} gene are very inefficient templates. In contrast, DNAs with mismatches outside the coding sequences are efficiently transcribed. A preliminary survey of the transcription of heteroduplexed 5SRNA genes suggests that a DNA with a mismatch within the internal control region is transcribed inefficiently, whereas, several with mismatches outside the internal control region are transcribed efficiently. These results are interpreted with respect to their implications for transcription factor binding and function.

METHODS

DNAs

M13 DNAs containing the *X. laevis* tRNA₁^{met} gene in both orientations (Met1 and Met2) and bisulfite-induced point mutants of each have been described (26). A similar collection of DNAs containing point mutants of the Syrian hamster 5SRNA gene (32) was prepared by R. Hart and M. Sullivan. An analysis of the transcription *in vitro* of selected 5SRNA gene mutants is described elsewhere (17).

Heteroduplexes were prepared by mixing 10 µg of RF M13-tRNA₁^{met} or M13-5SRNA DNA linearized with BglII at the unique site in M13 sequences with 5 µg of single stranded DNA in 200 µl of 0.1 M Tris·HCl, 1 mM EDTA, pH 7.5 and adding 100 µl of 1 N NaOH. After 5 min at room temperature, the solution was diluted with 200 µl of 0.2 M Hepes pH 7.5, 0.5 M HCl and the DNAs were annealed at 68°C for 90 min. The annealed DNAs were precipitated, dissolved in 10 mM Tris, pH 7.5, 1 mM EDTA at a concentration of 1 mg/ml and analyzed by gel electrophoresis to ensure that FII DNA had formed by renaturation of the denatured FIII strands and the single-stranded circles.

Transcription Analyses

DNAs to be analyzed for transcription were mixed with a reference DNA (for tRNAs, the reference DNA was pTH1, a pBR322 clone containing the Syrian hamster 5SRNA gene (32); for 5SRNAs, the reference DNA was M13tRNA₁^{met} DNA (26)), precipitated with ethanol and resuspended at 1 mg/ml in a solution containing 88 mM NaCl, 10 mM Tris·HCl pH7.4, 50 mM β-D-arabinofuranoside 5' triphosphate (araCTP) [α-³²P]GTP at 10 µCi/ml and the reference DNA at a concentration of 0.1 mg/ml. A 25 nl aliquot of this DNA solution was injected into nuclei of centrifuged oocytes (33). Following 3 hours

Figure 1: Persistence of DNAs in oocytes.

Odd-numbered lanes indicate DNAs recovered after three hour incubation in oocytes. Even-numbered lanes indicate DNAs before injection into oocytes.

incubation, the oocytes were harvested, the RNAs extracted and analyzed on 8 M Urea, 10% polyacrylamide gels (33).

Inclusion of araCTP in the injection buffer prevented conversion of single stranded DNA into duplex DNA within oocytes, a prerequisite for transcription by RNA polymerase III (34). Linear DNA is a poor template for RNA polymerase III, hence it does not contribute significantly to the product observed when reannealed, circular DNA is injected. Thus, it was possible to

inject the DNAs directly from a reannealing reaction, without having to purify the circular heteroduplex from the residual linear and single-stranded input DNAs.

To ascertain the fate of heteroduplexed DNAs within oocyte nuclei, we compared the stability of several heteroduplexes (prepared as described above) with supercoiled (Form I), nicked circular (Form II), linear (Form III) and single stranded M13 tRNA^{met}₁ DNAs. After injection of DNAs at a concentration of 1 mg/ml, the oocytes were incubated for 3 hr., and the nucleic acids extracted and deproteinized. Following treatment with RNase, the DNAs were fractionated by electrophoresis through 1% agarose gels, blotted to nitrocellulose and hybridized with labeled M13 tRNA^{met}₁ DNA.

The data in Fig. 1 indicate that single-stranded DNA is largely degraded (lanes 1 + 2). In previous studies, Forms I and II DNAs were found to be converted to relaxed circular, and then to become supercoiled (35). In this analysis, it is apparent that the mixture of Form I and Form II DNA injected into oocytes persists, but the relative amount of each form changes (lanes 5 + 6). Mixtures of denatured and reannealed DNAs containing single stranded, linear and Form II heteroduplexes prepared by annealing mutant B4 FIII DNA with wild type single stranded tRNA^{met}₁ DNA, or mutant Z65 single-stranded DNA with wild type tRNA^{met}₁ FIII DNA mirror the separate fates of each DNA in the mixture. Single stranded DNA appears to be degraded and FIII and FII DNAs persist (lanes 7-10).

RESULTS

Properties of tRNA^{met}₁ and 5SRNA gene mutants.

Previous studies with mutationally altered *X. laevis* tRNA^{met}₁ genes indicated that GC→AT transitions at positions 12, 29, 41, 49, 52, 56 and 61 reduced expression of tRNA^{met}₁ in oocytes at least tenfold (26). It is likely that many of these mutations affect binding or function of either or both the transcription factors required to form a stable transcription complex with tRNA^{met}₁ DNA, TFIIIC and TFIIIB (1-2). Direct measurements of the binding of a HeLa cell fraction containing TFIIIC indicates that virtually the entire tRNA^{met}₁ coding sequence is protected from DNase 1 cleavage by protein(s) (5).

The effects of mutations in the tRNA^{met}₁ gene upon transcription depend upon the assay system that is used (22). Employing a crude germinal vesicle lysate from *X. laevis* oocytes (36), we have observed that most of these point mutants are transcribed in vitro, in contrast to their failure to be expressed in oocytes (Sullivan and Folk, unpublished observations).

Figure 2: Transcription in oocytes of heteroduplex and homoduplex tRNA^{met}₁ DNA.

Autoradiograph of polyacrylamide gels displaying transcription products of DNAs injected into oocytes. tRNA^{met}₁ precursor and mature products migrate to the lower portion of the gel. 5SRNA transcribed from pTH1DNA (32) included as an internal control migrates near the top.

Presumably, the large difference in the ratio of transcription factors to functional templates between the oocyte and in vitro assay systems accounts for this difference in transcriptional activity. Direct measurement of the binding of purified TFIIIB and TFIIIC proteins to the mutant tRNA genes should resolve how these G·C→A·T transitions affect formation or some subsequent function of the transcription complex. A recent study of the binding of yeast TFIIIC proteins to tRNA^{tyr} mutants indicated that mutations in the intragenic 3' proximal sequences (the "B block") affect binding, but mutations in the intragenic 5' proximal sequences have only a small effect upon binding and may alter transcription in some other way (31).

Analysis of the expression of 5SRNA genes in *X. laevis* oocytes indicates that point mutations at many locations in the gene have little effect upon transcription (17). However, assays of transcription or transcription factor binding in vitro with some of these same mutants indicate that the capacity of TFIIIB and TFIIIC to form functional preinitiation complexes is altered (17). An extensive analysis of the transcription in vitro of similar 5SRNA gene point mutants has recently been reported (15,16).

Transcription of tRNA^{met} gene heteroduplexes.

To further explore the effect upon transcription of altering individual base pairs in the tRNA^{met} gene, we constructed heteroduplexes between DNAs containing single G·C→A·T transitions and unmutated strands, and measured their capacity to serve as templates in *X. laevis* oocytes. Together with the circular heteroduplex DNA injected into oocytes were smaller amounts of the linear and single stranded DNAs which had not renatured to form a heteroduplex. Injection of araCTP with these DNAs prevented the single stranded DNA from being converted to duplex DNA, a necessary prerequisite for transcription by RNA polymerase III (34). The linear, duplex DNA which did not form heteroduplex is a poor template (see below). The injected heteroduplexes contain the mutated nucleotide on the noncoding strand, so that any RNA transcripts should have a wild type sequence and normal stability.

Surprisingly, every heteroduplex containing a mismatch within the coding region of tRNA^{met} we examined was poorly transcribed or not transcribed at all (Fig. 2 and Fig. 3). Heteroduplexes of DNAs with mutations near the ends of the coding sequences were transcribed, albeit poorly, in some experiments, and not at all in other experiments. Heteroduplexes of DNAs with mismatches outside the coding sequences, however, were transcribed normally. These results indicate that some property of a DNA containing a T·G or A·C base mismatch within the sequence transcribed by RNA polymerase III renders transcription exceedingly inefficient in vivo. Even sites at which transition mutations in normal duplex DNA have no pronounced effect upon transcription (positions 2,24,32,33,34,71; Fig. 3), when mispaired profoundly reduced transcription, suggesting that it may not be faulty binding of DNA per se by TFIIIC or TFIIIB that impairs transcription of the heteroduplex DNAs.

Figure 3: Summary of transcriptional analyses of tRNA^{met}₁ gene. The duplex DNA sequence encoding mature tRNA^{met}₁ (nt 1-73) and several 5' upstream bases is provided, together with mutations in DNAs whose transcription in oocytes was reported previously (26). Arrows point to bases present in heteroduplex DNAs whose transcription was analyzed in this study. A summary of transcription analyses in oocytes of heteroduplex and homoduplex DNAs is given at top of figure.

Transcription of 5SRNA gene heteroduplexes.

To determine the generality of the transcriptional incompetence of DNA heteroduplexes, we constructed several 5SRNA gene heteroduplexes and measured their transcription in oocytes (Fig. 4). Similarly to what we observed with the tRNA^{met}₁ mutants, a heteroduplex DNA with a mismatch in the internal control region (at position 64) was not transcribed. Transition mutations at this site, in homoduplex DNAs, have no effect upon transcription in oocytes (17). However, 5SRNA DNAs with mismatches in 5' proximal intragenic sequences (but outside the internal control region, at positions 1, 7, and 27) were transcribed nearly as well as duplex DNA. A heteroduplex with a mismatch outside the 5S gene (at position +9 from the 3' end) was also transcribed well.

DISCUSSION

At present we can only conjecture about the failure of most DNAs with mismatches in their intragenic control regions to be transcribed in oocytes. The G-T and A-C mismatches generated by annealing mutant and wild type DNAs are unlikely to markedly alter DNA structure (37,38,40,41); however, they decrease the local stability of the duplex (37-41). Some sites within the tRNA^{met}₁ gene have little if any effect upon transcription when mutated, but greatly diminish transcription when present as mismatches (where the coding strand is wild type and the noncoding strand has the same nucleotide as a DNA with a transition mutation). Thus, it seems unlikely that transcription factors or RNA polymerase fail to bind because of these mismatches. This argument is even more compelling when one recalls that insertion of HindIII linkers in tRNA^{met}₁ at positions 32 or 48, or substitution of sequences between positions 32-47 with several HindIII linkers do not have nearly as great an effect upon transcription (22) as do mismatches at positions 32, 33, or 34 (Fig. 2). We are preparing to measure directly the binding of

Figure 4: Transcription in oocytes of heteroduplex and homoduplex 5SRNA DNAs.

Autoradiograph of a polyacrylamide gel displaying transcription products of DNAs injected into oocytes. 5S RNAs migrate near the top of the gel section while $tRNA^{met}$ transcribed from met1 DNA included as an internal control migrates near the bottom. Position of mutant (heteroduplex) sites within the coding sequence of the Syrian hamster 5S RNA gene (32) are indicated above the columns.

transcription factors to these $tRNA_1^{met}$ constructs, and to extend the analysis of the transcription of heteroduplex 5S genes.

Because the most pronounced effect of these mismatches is on the stability of the duplex, we believe that the failure of DNAs with mismatches to be transcribed reflects an aspect of the transcription process involving denaturation and reassociation of the template. During prokaryotic transcription, the template is denatured and subsequently renatured, with approximately 17 bp of open duplex introduced by *E. coli* RNA polymerase (42). If a similar process occurs during transcription by RNA polymerase III, renaturation of the duplex may be impeded by the destabilizing effect of the mismatch. Both TFIIC and TFIIIA bind single stranded DNA, and could further alter the equilibrium between single stranded and duplex DNA (43,44). It is noteworthy that sites within the $tRNA_1^{met}$ and 5SRNA genes which may not

interact with transcription factors, when present as mismatches, have little effect upon transcription. This suggests that the local instability of the base mismatch has its most pronounced effect when the DNA is complexed with proteins whose function is to aid the process of transcription.

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REFERENCES

1. Shastry, B.S., Ng, S.Y. and Roeder, R.G. (1982) *J. Biol. Chem.* 257, 12979-12986.
2. Lassar, A.B., Martin, P.L. and Roeder, R.G. (1983) *Science* 22, 740-748.
3. Bieker, J.J., Martin, P.L. and Roeder, R.G. (1985) *Cell* 40, 119-127.
4. Setzer, D.R. and Brown, D.D. (1985) *J. Biol. Chem.* 260, 2483-2492.
5. Carey, M.R., Gerrard, S.P. and Cozzarelli, N.R. (1986) *J. Biol. Chem.* 261, 4309-4317.
6. Schlissel, M.J. and Brown, D.D. (1984) *Cell* 37, 903-913.
7. Gargiulo, G., Razvi, F. and Worcel, A. (1984) *Cell* 38, 511-521.
8. Gottesfeld, J. and Bloomer, L.S. (1982) *Cell* 28, 781-791.
9. Engelke, D.R., Ng, S.Y., Shastry, B.S. and Roeder, R.G. (1980) *Cell* 19, 717-728.
10. Sakonju, S., Bogenhagen, D.F. and Brown, D.D. (1980) *Cell* 19, 19-35.
11. Bogenhagen, D.F., Sakonju, S. and Brown, D.D. (1980) *Cell* 19, 27-35.
12. Sakonju, S. and Brown, D.D. (1982) *Cell* 31, 395-405.
13. Bogenhagen, D.F. (1985) *J. Biol. Chem.* 260, 6466-6471.
14. Ciliberto, G., Rauegi, G., Constanzo, F., Dente, L. and Cortese, R. (1983) *Cell* 32, 725-732.
15. Pieler, T., Appel, B., Oei, S.L., Mentzel, H. and Erdmann, A. (1985) *EMBO Journal* 4, 1847-1853.
16. Pieler, T., Oei, S.L., Hamm, J., Engelke, U. and Erdmann, A. (1985) *EMBO Journal* 4, 3751-3756.
17. Shastry, B.S., Greenstein, D., Roeder, R.G., Hart, R.P., Datta, M.P., Sullivan, M. and Folk, W.R. (1987) Manuscript in preparation.
18. Klemenz, R., Stillman, D. and Geiduschek, E.P. (1982) *Proc. Nat. Acad. Sci. USA* 79, 6191-6195.
19. Stillman, D. and Geiduschek, E.P. (1984) *EMBO Journal* 3, 847-853.
20. Fuhrman, S.A., Engelke, D.R. and Geiduschek, E.P. (1984) *J. Biol. Chem.* 259, 1934-1943.
21. Camier, S., Gabrielsen, O., Baker, R. and Sentenac, A. (1985) *EMBO Journal* 4, 491-500.
22. Hofstetter, H., Kressman, A. and Birnstiel, M.L. (1981) *Cell* 24, 573-585.
23. Ciliberto, G., Castagnoli, L. and Cortese, R. (1983) *Current Topics in Developmental Biology*, Vol. 18, pp. 59-88.
24. Sharp, S., DeFranco, D., Dingermann, T., Farrell, P. and Soll, D. (1981) *Proc. Nat. Acad. Sci. USA* 78, 6657-6661.
25. Galli, G., Hofstetter, H. and Birnstiel, M. (1981) *Nature* 294, 626-631.
26. Folk, W.R. and Hofstetter, H. (1983) *Cell* 33, 585-593.
27. Allison, D.S., Goh, S.H. and Hall, B.D. (1983) *Cell* 34, 655-664.
28. Newman, P.J., Ogden, R.C. and Abelson, J. (1983) *Cell* 35, 117-125.
29. Traboni, C., Ciliberto, G. and Cortese, R. (1984) *Cell* 36, 179-187.

30. Stewart, T., Soll, D. and Sharp, S. (1985) *Nucleic Acids Res.* 13, 435-447.
31. Baker, R.E., Gabrielsen, O. and Hall, B.D. (1986) *J. Biol. Chem.* 261, 5275-5282.
32. Hart, R.P. and Polk, W.R. (1982) *J. Biol. Chem.* 257, 11706-11711.
33. Kressman, A., Clarkson, S.G., Telford, J.L. and Birnstiel, M. (1977) *Cold Spring Harbor Symp. Quant. Biol.* 42, 1077-1082.
34. Cortese, R., Harland, R. and Melton, D. (1980) *Proc. Nat. Acad. Sci. USA* 77, 4147-4151.
35. Wyllie, A.H., Laskey, R.A., Finch, J. and Gurdon, J. B. (1978) *Developmental Biology* 64, 178-188.
36. Birkenmeier, E.H., Brown, D.D. and Jordan, E. (1978) *Cell* 15, 1077-1086.
37. Patel, D.J., Kozlowski, S.A., Marky, L.A., Rice, J.A., Broka, C., Dallas, J., Itakura, K. and Breslauer, K.J. (1982) *Biochemistry* 21, 437-444.
38. Chuprina, V. and Poltev, V.I. (1985) *Nucleic Acids Res.* 13, 141-154.
39. Werntges, H., Steger, G., Riesner, D. and Fritz, H-J. (1986) *Nucleic Acids Res.* 14, 3773-3798.
40. Brown, T., Hunter, W.N., Kneale, G. and Kennard, O. (1986) *Proc. Nat. Acad. Sci. USA* 83, 2402-2406.
41. Hunter, W.N., Brown, J., Anand, N.N and Kennard, O. (1986) *Nature (London)* 320, 552-555.
42. Gamper, H.B. and Hearst, J.E. (1982) *Cell* 29, 81-90.
43. Hanas, J., Hazuda, D. and Wu, C.W. (1985) *J. Biol. Chem.* 260, 13316-13320.
44. Stillman, D.J., Caspers, P. and Geiduschek, E.P. (1985) *Cell* 40, 311-317.