Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 3978–3982, June 1985
Biochemistry

Opposite-strand RNAs from the 5′ flanking region of the mouse dihydrofolate reductase gene

(eukaryotic promoters/small nuclear RNAs)

PEGGY J. FARNHAM, JOHN M. ABRAMS, AND ROBERT T. SCHIMKE

Department of Biological Sciences, Stanford University, Stanford, CA 94305

Contributed by Robert T. Schimke, February 12, 1985

ABSTRACT **We have characterized a transcription unit in the 5′ flanking region of the mouse gene encoding dihydrofolate reductase (EC 1.5.1.3) that is oriented in the opposite direction to that of dihydrofolate reductase transcription. These opposite-strand RNAs are 180–240 nucleotides long, differing in length at their 5′ ends. They are abundant in nuclear RNA and are not polyadenylylated. We suggest that the promoter region of the dihydrofolate reductase gene functions in a bidirectional manner to produce both these RNAs and dihydrofolate reductase mRNAs.**

Dihydrofolate reductase (DHFR; tetrahydrofolate dehydrogenase, EC 1.5.1.3) belongs to a class of "housekeeping" enzymes, whose products are necessary for growth of all cell types. Specifically, DHFR is a key enzyme in the *de novo* synthesis of glycine, purines, and thymidylate and is thus involved in DNA replication. Previous studies have shown that DHFR is cell cycle regulated at the transcriptional level (1, 2). Although it is known that the burst of transcription at the G_1/S boundary results from enhancement of transcription from a constitutive promoter (2), the mechanism controlling this cyclic increase and decrease in transcription rate is unknown.

The 1000 base pairs (bp) of DNA preceding the *DHFR* translation start codon are composed of a complex pattern of regulatory sequences (3). The region between −101 and −272 consists of 3½ copies of a 48-bp sequence. These repeats contain a sequence CACAAATA, which resembles a combination of the "CAAT" and "TATA" boxes that are utilized as RNA polymerase II transcription signals by many eukaryotic genes (4). Each repeat also contains the hexanucleotide GGGCGG, six copies of which are also present in the 21-bp repeats of the simian virus 40 (SV40) promoter region (5). In addition, homology to the 21-bp repeats of SV40 is found between −559 and −594. The only sequences in this 1000 bp that resemble a characteristic RNA polymerase II promoter are CAACT at −748 and TAATAA at −673. However, no cellular RNAs have been identified which utilize this TATA box (2, 3). Instead, the major site of transcription initiation is in the most 3′-ward 48-bp repeat.

Comparison of the 5′ flanking sequences of the human and mouse *DHFR* genes reveals that the majority of the 1000 bp 5′ of the start codon are evolutionarily conserved (6). Since it is likely that this region is involved in the regulation of the *DHFR* gene, we have pursued a more detailed characterization of its transcription pattern. In the process of studying *DHFR* transcription, we identified a series of RNA transcripts originating from the opposite strand to that coding for DHFR mRNA. These opposite-strand RNAs are small, nuclear, poly(A)$^-$ RNAs whose 5′ ends map near the *DHFR* promoter region.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

DNA Constructions. The recombinant DNA plasmid pSP65-RT10+ (a gift from C. Gasser) contains a 1017-bp *Eco*RI/*Hin*dIII fragment derived from the 5′ end of the mouse *DHFR* gene and inserted into the *Eco*RI and *Hin*dIII sites of plasmid pSP65 (Promega Biotec, Madison, WI). The insert contains 1006 nucleotides upstream from the translation start site, 6 nucleotides of coding sequence, and 5 nucleotides of pBR322 sequence (see Fig. 1). The recombinant DNA plasmid pSP65-ST+ was created by digestion of pSP65-RT10+ with *Eco*RI and *Sma* I. The linear plasmid, now containing only the 416-bp *Sma* I to *Hin*dIII insert, was isolated in SeaPlaque agarose, and the ends were filled by using the Klenow fragment of DNA polymerase and ligated with T4 DNA ligase (7). The ligation products were used to transform *Escherichia coli* strain HB101 according to standard protocols (7, 8). Plasmid DNA was obtained by standard procedures (9).

Cells and Culture Conditions. The mouse cell lines used in these studies include 3T6 cells (10) and the methotrexate-resistant cell lines 3T6 R50-MS6-clone A (2), 3T6 R400 (11) and S180 M500 (12). These cell lines contain amplified *DHFR* genes (see legend of Fig. 5) and were maintained as described in the previous studies.

RNA Protection Experiments. Cytoplasmic RNA was prepared by the method of Maniatis *et al.* (13). The nuclear pellets were used to make nuclear RNA. Total cellular and nuclear RNA were prepared by the guanidinium isothiocyanate/cesium chloride method as described by Setzer *et al.* (12). Preparation of R400 total, poly(A)$^+$, and poly(A)$^-$ RNA (gifts from C. Gasser) was as described by Gasser (14). All RNA preparations were electrophoresed on formaldehyde gels (13) and visualized by ethidium bromide staining to corroborate spectrophotometric quantification and to ensure that no degradation had occurred.

To examine RNAs from the 5′ region of the *DHFR* gene, the SP6 promoter system (Promega Biotec) was used. Plasmids SP65-RT10+ and SP65-ST+ produce RNA transcripts having the same polarity as *DHFR* mRNAs and thus are complementary to transcripts coded from the opposite strand to *DHFR*. SP65-RT10+ was linearized to produce run-off transcripts complementary to the entire *Eco*RI to *Hin*dIII region, the *Eco*RI to *Xho* II region, or the *Eco*RI to *Stu* I region (see Fig. 1). SP65-ST+ was linearized to produce a run-off transcript complementary to the *Sma* I to *Hin*dIII region.

Approximately 0.5–2 × 10^6 cpm of RNA probe and 10 μg of cellular RNA were used in each RNA protection experiment, performed according to the Promega Biotec protocols with the following modifications. Hybridization of the RNA and probe was as described in Favaloro *et al.* (15). After hybridization, 300 μl of RNase digestion buffer (Promega

Abbreviations: DHFR, dihydrofolate reductase; bp, base pair(s); SV40, simian virus 40.

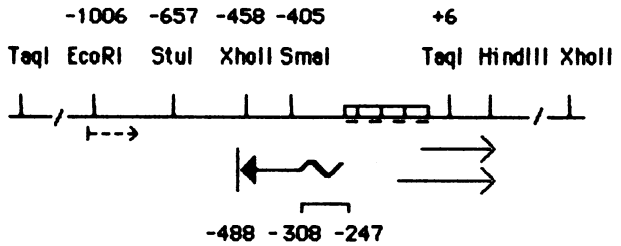

FIG. 1. The 5′ end of the *DHFR* gene. This diagram represents the 1000-bp insert from the 5′ region of the *DHFR* gene and flanking vector sequences of the plasmid pSP65-RT10+. The nucleotide sequence is numbered such that the first base of the translation initiation codon used in the synthesis of DHFR is numbered +1. The location of restriction sites used in cloning, S1 nuclease reactions, and SP6 promoter transcriptions are indicated. Only restriction sites derived from murine DNA are numbered. Transcription initiation from the SP6 promoter occurs 6–9 bp upstream of the *Eco*RI site. The site of SP6 initiation and the direction of transcription are indicated by a dashed arrow. The 5 bp between the *Taq* I and *Hin*dIII sites are the nucleotides in pBR322 from the *Cla* I to the *Hin*dIII sites. The 48-bp repeats (boxes) and the GGGCGG hexanucleotides (underlines) of the *DHFR* promoter region are also shown. The major *DHFR* transcripts are indicated by open arrowheads, whereas the opposite-strand transcripts are indicated by the closed arrowhead. The heterogeneity of the 5′ ends of the opposite-strand transcripts is represented by the waved line. The complete nucleotide sequence of this region is presented elsewhere (3).

Biotec) containing RNase A at 1 μg/ml and RNase T1 at 0.03 unit/ml was added to each sample, followed by digestion at 30°C for 30 min. The reaction was terminated and the samples were processed according to the Promega Biotec protocol.

A 1485-bp *Xho* II fragment spanning the promoter region was used as a DNA probe in an S1 nuclease reaction to map the 5′ ends of the opposite-strand RNAs. The 1584-bp *Taq* I fragment spanning the promoter region was used as the DNA probe in S1 nuclease experiments examining the *DHFR* transcription levels. The plasmid SP65-RT10+ was digested with either *Xho* II or *Taq* I, 5′-end-labeled with [γ-^{32}P]ATP by using polynucleotide kinase (Bethesda Research Laboratories), separated by agarose gel electrophoresis, and purified from the gel. Approximately 20,000 cpm of DNA probe and 25 μg of cellular RNA were used in each reaction. S1 nuclease reactions were performed according to the method of Favaloro *et al.* (15) as modified from Berk and Sharp (16). Samples from both the RNA and DNA probe protection experiments were analyzed on 5% or 8% polyacrylamide/8 M urea gels (13).

RESULTS

Identification of Opposite-Strand Transcripts. We have located a transcription unit in the 5′ region of the mouse *DHFR* gene. Using a uniformly labeled *Eco*RI/*Hin*dIII RNA probe produced by the SP6 promoter system (see Fig. 1 for a map of the 5′ end of the *DHFR* gene), we detected cellular RNAs transcribed from the strand opposite to that used for the *DHFR* gene. As can be seen in Fig. 2, lane 2, this cluster of RNAs ranges from 180 to 240 nucleotides in length. Since a uniformly labeled probe was used, the protected fragments could lie anywhere in the 1012 bp of *DHFR* flanking sequence. To more precisely map their location, a series of progressively shorter probes was used. Along with the 1017-nucleotide *Eco*RI/*Hin*dIII probe, a 548-nucleotide *Eco*RI/*Xho* II probe, and a 349-nucleotide *Eco*RI/*Stu* I probe were used in protection experiments, the results of which are shown in Fig. 3. The *Eco*RI/*Hin*dIII probe (lane 1) shows the series of protected fragments seen in Fig. 2. However, when the probe is shortened to 548 nucleotides, a 30-nucleotide fragment is seen (lane 2). The shortest probe (349-nucleotide *Eco*RI/*Stu* I) results in only undigested probe, indicating no protection of this fragment (lane 3). This allows the 3′ end of the RNA to be located to approximately position −488 (see Fig. 1). With a 3′ end fixed to position −488 and knowing the lengths of the protected fragments from the longer probe, it was possible to map the 5′ ends to the region of −248 to −308. The heterogeneity seen with the long probe is not present when the *Eco*RI/*Xho* II probe is used, implying that it could be due to the 5′ end of the RNA and not the 3′ end. To more accurately place the 5′ ends of the RNAs, two other experiments were performed. First, the *Eco*RI/*Sma* I fragment was removed from the SP65-RT10+ plasmid, producing plasmid SP65-ST+. The uniformly labeled *Sma* I/*Hin*dIII probe made from this plasmid should produce protected fragments having a 3′ end fixed at −405. Protection experiments using this probe gave heterogeneous fragments that were correspondingly shorter than the full length (Fig. 3, lane 5), placing the heterogeneous 5′ ends from −253 to −298. S1 nuclease protection analysis was also used to map the 5′ ends. The 1485-bp *Xho* II fragment span-

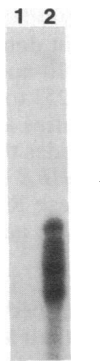

FIG. 2. Identification of opposite-strand transcripts. RNA protection experiments using a uniformly labeled *Eco*RI/*Hin*dIII probe and parental 3T6 nuclear RNA (lane 1) or 3T6 R50 total RNA (lane 2) are shown. 3T6 R50 cells contain 40-50 times as many *DHFR* genes as do the parental cells. Longer exposure of the autoradiogram detects the same protected fragments in the parental 3T6 cells. The protected fragments seen in lane 2 range from 180 to 240 nucleotides in length and were sized by comparison to known DNA markers.

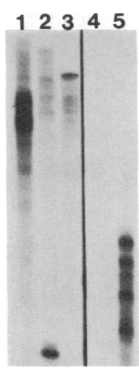

FIG. 3. Transcript mapping using uniformly labeled RNA probes. Location of the 3′ end of the small RNAs is demonstrated with M500 total RNA in lane 1 (1017-nucleotide *Eco*RI/*Hin*dIII probe), lane 2 (548-nucleotide *Eco*RI/*Xho* II probe), and lane 3 (349-nucleotide *Eco*RI/*Stu* I probe). Protection experiments using the 416-nucleotide *Sma* I/*Hin*dIII probe are shown in lane 4 (parental 3T6 nuclear RNA) and lane 5 (3T6 R50 total RNA). Lanes 1–3 are from a 5% polyacrylamide gel, whereas lanes 4 and 5 are from an 8% polyacrylamide gel. Fragment lengths were determined by comparison to DNA markers on each gel.

ning the start sites was 5′-end-labeled and used in an S1 nuclease protection experiment demonstrating that the 5′ ends represent a ladder of about 10-nucleotide increments (Fig. 4, lane 4) in the region from −253 to −303. This combination of mapping experiments identifies a group of RNAs ranging in size from 180 to 240 nucleotides that are transcribed from the 5′ flanking region of the *DHFR* gene in the direction opposite to *DHFR* mRNAs. These RNAs are colinear with heterogeneous 5′ ends beginning adjacent to the promoter region for the *DHFR* gene.

Characterization of the RNA. To ensure that the RNAs we were detecting were transcribed from the 5′ region of the *DHFR* gene, we compared their levels in various DHFR-amplified cell lines to those in nonamplified cells. Using the uniformly labeled *Eco*RI/*Hin*dIII probe in an RNA protection experiment, we compared 3T6 cells (Fig. 5, lane 1) to R50 (lane 2), R400 (lane 3), and M500 (lane 4) cell lines. The amount of the RNAs increases with amplification, indicating that they are transcribed from the amplified unit. RNA from a DHFR-deficient Chinese hamster ovary cell line (17) that contains a transfected murine *DHFR* minigene with a truncated 5′ flanking region (14) was also used in an RNA protection experiment. This amplified cell line should not produce the murine opposite-strand RNAs since it is truncated at −160. As expected, the protected fragments were not seen (data not shown).

We next examined the cellular distribution of these RNAs. R50 RNA was fractionated into cytoplasmic and nuclear components. To compare the localization of the opposite-strand RNAs to that of *DHFR* mRNAs, S1 nuclease reactions were performed, using a 1584-bp *Taq* I probe to measure *DHFR* mRNA levels (Fig. 4, lanes 1 and 2) and a 1485-bp *Xho* II probe to measure opposite-strand RNA levels (lanes 3 and 4). *DHFR* mRNA is present in both the cytoplasmic (lane 1) and nuclear (lane 2) fractions. The small RNAs, in contrast, are present only in the nuclear (lane 4) fraction and not in the cytoplasmic fraction (lane 3). The *DHFR* mRNA lanes serve as a control to show that the cytoplasmic RNA is intact and allow a comparison of the amounts of the different transcripts. It appears that these opposite-strand transcripts are produced from the amplified units in approximately the same levels as are the *DHFR* mRNAs.

We also examined R400 total RNA that had been separated into poly(A)$^+$ and poly(A)$^-$ fractions. These RNAs have been used in a blot hybridization analysis to show that the DHFR messages are present in the poly(A)$^+$ fraction and not in the poly(A)$^-$ fraction (14). Fig. 6 shows an RNA protection experiment using these RNAs and the *Eco*RI/*Hin*dIII probe. The opposite-strand RNAs are present only in total RNA (lane 1) and poly(A)$^-$ RNA (lane 3) and not in poly(A)$^+$ RNA (lane 2).

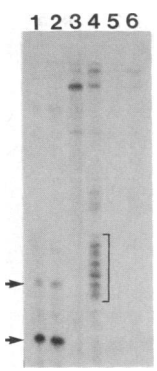

FIG. 4. S1 nuclease mapping of RNA transcripts. The 1584-bp *Taq* I fragment was used to identify *DHFR* transcripts in 3T6 R50 cytoplasmic RNA (lane 1), 3T6 R50 nuclear RNA (lane 2), and 3T6 nuclear RNA (lane 5). The 1485-bp *Xho* II fragment was used to identify opposite-strand RNAs in 3T6 R50 cytoplasmic RNA (lane 3), 3T6 R50 nuclear RNA (lane 4), and 3T6 parental nuclear RNA (lane 6). Fragments representing the major and secondary *DHFR* initiation sites are marked with arrows. The opposite-strand RNAs are indicated by a bracket. The larger fragments seen in lane 4 are probe-specific S1 nuclease artifacts.

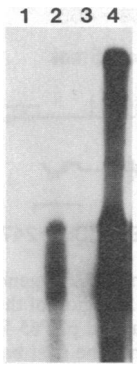

FIG. 5. Quantitation of the opposite-strand RNAs in amplified DHFR cell lines. The 1017-nucleotide *Eco*RI/*Hin*dIII uniformly labeled RNA probe was used to quantitate the levels of the opposite-strand RNAs in 3T6 cells (lane 1), 3T6 R50 cells containing 50 copies of the *DHFR* genes (lane 2), 3T6 R400 cells containing 15 copies of the *DHFR* genes (lane 3), and S180 M500 cells containing 500 copies of the *DHFR* genes. When the autoradiogram was exposed for a much longer time, the opposite-strand RNAs could be detected in the unamplified cells (not shown).

DISCUSSION

We have identified small nuclear poly(A)$^-$ transcripts originating from the 5′ flanking region of the *DHFR* gene and transcribed in the direction opposite to that of *DHFR* mRNAs. These RNAs range in size from 180 to 240 nucleotides and have heterogeneous 5′ ends originating adjacent to the *DHFR* promoter region. The *DHFR* promoter consists of 3½ tandem copies of a 48-bp repeat that contain the hexanucleotide GGGCGG characteristic of the 21-bp repeats of the SV40 promoter. The 21-bp repeat functions as an SV40 promoter element *in vivo* for both the early and late transcripts (18–20) and can be inverted without losing its ability to direct early RNA synthesis (21). In the absence of contiguous SV40 transcription control sequences, the 21-bp repeats are capable of initiating transcription in a bidirectional manner from

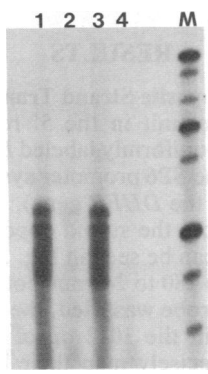

FIG. 6. Localization of the small RNAs. A uniformly labeled 1017-nucleotide *Eco*RI/*Hin*dIII probe was used to determine the abundance of the opposite-strand RNAs in 3T6 R400 total RNA (lane 1), 3T6 R400 poly(A)$^+$ RNA (lane 2), and 3T6 R400 poly(A)$^-$ RNA (lane 3). Lane 4 shows the level in 3T6 nuclear RNA. The DNA markers (lane M) are *Hin*fI fragments from pDHFR11 (ref. 12).

pBR322 sequences in an *in vitro* transcription assay (22). This characteristic of the SV40 promoter, that of one region directing synthesis of RNA bidirectionally on opposite strands, appears to be duplicated by the *DHFR* promoter.

The 5′ heterogeneity of these small RNAs is likely due to the unique structure of the *DHFR* promoter, which has also been shown to direct multiple initiations in the *DHFR* mRNAs (2, 3). Other RNA polymerase II promoters that do not have a TATA box produce heterogeneous starts, including the polyoma late promoter (23), the adenovirus 2 DNA-binding protein promoter (24), and the SV40 late promoter (25). In addition, numerous studies have shown that elimination or alteration of the TATA box causes specific transcription initiation to degenerate to many sites (18, 26–29). The function of the TATA box may be to confer a fixed unidirectional initiation site for RNA polymerase II. Promoter regions lacking this sequence may function bidirectionally and produce heterogeneous initiations.

Although we do not know if these RNAs are functional or just fortuitous transcripts that result from an unusual promoter region, all evidence indicates that they are not part of a longer transcript. The opposite-strand RNAs cannot represent a complete intron since no adjacent exon sequences are detected in total or poly(A)$^+$ RNA in RNA protection experiments employing probes extending both 5′ and 3′ from the region of the small RNAs. It remains possible that these RNAs represent highly stable degradation products from splicing intermediates of a large mRNA originating within the amplified DNA segments. We think this is unlikely since lariat structure intermediates of mRNA splicing are present in the nucleus in extremely small amounts (30). In contrast, the small nuclear RNAs are present in an abundance comparable to that of cytoplasmic *DHFR* mRNA. Also, the sequence of the DNA coding for the small RNAs is highly homologous between the mouse and the human *DHFR* genes. Inasmuch as intervening sequences in homologous genes (including the mouse and human *DHFR* genes) characteristically have low homology, this argues against these RNAs representing an intron degradation product. Conversely, these RNAs could constitute exon-like segments of a larger mRNA species that are spliced at such a rapid rate that the primary transcript is never observed. However, since these RNAs are limited to the poly(A)$^-$ fraction of nuclear RNA, the putative spliced transcript could not be a classical mRNA. Lastly, hybridization analyses using double-stranded DNA probes up to 90 kbp 5′ or 46 kbp 3′ of the small RNAs do not detect amplified RNAs in total poly(A)$^+$ M500 RNA other than those coding for DHFR (N. Federspiel, personal communication). For all of the above reasons, we do not believe that the small RNAs constitute degradation or splicing products. When the small RNA probes are used in blot hybridization analysis, their G+C-richness (>65%) results in weak hybridization to rRNA and to small RNAs (200–300 nucleotides), but no poly(A)$^+$ RNAs hybridize as strongly (data not shown). Our inability to obtain a strong hybridization signal in hybridization analysis may be influenced by stable secondary structures in the small RNA probe (see below).

Several classes of small RNAs are considered to be important in gene regulation. Birchmeier *et al.* (31) have shown that the accurate formation of sea urchin histone H3 mRNA termini requires a 60-nucleotide nuclear RNA. Another class, the U-snRNAs are small, nuclear, poly(A)$^-$ transcripts originating from non-TATA box RNA polymerase II promoters (32). The U-snRNAs have the potential to form multiple stable secondary structures and have been implicated in the recognition of 5′ and 3′ splice sites (33, 34). The RNAs identified in this report have many of the properties of the U-snRNAs, including the potential to form multiple stable stem-and-loop structures. In fact, 10 such overlapping stems were identified by using the criterion of a ΔG of formation greater than -20 kcal/mol, using the Queen and Korn program (35). Intramolecular base pairing is possible over most of the length of these RNAs. Small RNAs are also utilized in antisense regulation. In these cases, RNAs complementary to prokaryotic gene transcripts have been identified (36, 37). The possibility that endogenous antisense regulation may be one of the normal components of gene regulation in eukaryotes has been raised by Izant and Weintraub (38). They have shown that artificially constructed antisense transcripts of the herpes simplex virus thymidine kinase (TK) gene can diminish the appearance of viral *TK* gene activity in mouse TK$^-$ L cells. Comparison of the opposite-strand RNAs to the *DHFR* gene and flanking sequences revealed a complementarity to the first 10 nucleotides of the major *DHFR* transcript and to a short region of the *DHFR* mRNA immediately following the translation stop codon. Although the biological significance of these homologies is untested, it is possible that base pairing between transcripts could influence *DHFR* regulation.

Note Added in Proof. Primer extension analyses have corroborated the protection experiments that map the 5′ ends of the opposite-strand RNAs to the DHFR promoter region.

We thank Shelley Sazer and Thea Tlsty for careful reading of this manuscript and Chuck Gasser for the generous gift of R400 RNA. This research was funded by a grant from the National Institutes of Health to R.T.S. (CA 16318). P.J.F. is a recipient of a National Institutes of Health Postdoctoral Fellowship and J.M.A. is a recipient of a National Science Foundation Graduate Fellowship.

1. Santiago, C., Collins, M. & Johnson, L. F. (1984) *J. Cell. Physiol.* **118**, 79–86.
2. Farnham, P. J. & Schimke, R. T. (1985) *J. Biol. Chem.*, in press.
3. McGrogan, M., Simonsen, C. C., Smouse, D. T., Farnham, P. J. & Shimke, R. T. (1985) *J. Biol. Chem.* **260**, 2307–2314.
4. Breathnach, R. & Chambon, P. (1981) *Annu. Rev. Biochem.* **50**, 349–383.
5. Byrne, B. J., Davis, M. S., Yamaguchi, J., Bergsma, D. J. & Subramanian, K. N. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 721–725.
6. Chen, M.-J., Shimada, T., Moulton, A. D., Cline, A., Humphries, R. K., Maizel, J. & Nienhuis, A. W. (1984) *J. Biol. Chem.* **259**, 3933–3943.
7. Crouse, G. F., Frischauf, A. & Lehrach, H. (1983) *Methods Enzymol.* **101**, 78–89.
8. Dagert, M. & Ehrlich, S. D. (1979) *Gene* **6**, 23–28.
9. Davis, R. W., Botstein, D. & Roth, J. R. (1980) *Advanced Bacterial Genetics* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
10. Brown, P. C., Beverley, S. M. & Schimke, R. T. (1981) *Mol. Cell. Biol.* **1**, 1077–1083.
11. Haber, D. A. & Schimke, R. T. (1981) *Cell* **26**, 355–362.
12. Setzer, D. R., McGrogan, M., Nunberg, J. H. & Schimke, R. T. (1980) *Cell* **22**, 361–370.
13. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).
14. Gasser, C. (1985) Dissertation (Stanford University, Stanford, CA).
15. Favaloro, J., Treisman, R. & Kamen, R. (1980) *Methods Enzymol.* **65**, 718–749.
16. Berk, A. J. & Sharp, P. A. (1977) *Cell* **12**, 721–732.
17. Urlaub, G. & Chasin, L. A. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4216–4220.
18. Fromm, M. & Berg, P. (1983) *J. Mol. Appl. Gen.* **2**, 127–135.
19. Fromm, M. & Berg, P. (1982) *J. Mol. Appl. Gen.* **1**, 457–481.
20. Hartzell, S. W., Byrne, B. J. & Subramanian, K. N. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 23–27.
21. Everett, R. D., Baty, D. & Chambon, P. (1983) *Nucleic Acids Res.* **11**, 2447–2464.
22. Mishoe, H., Brady, J. N., Radonovich, M. & Salzman, N. P. (1984) *Mol. Cell. Biol.* **4**, 2911–2920.

23. Jat, P., Roberts, J. W., Cowie, A. & Kamen, R. (1982) *Nucleic Acids Res.* **10,** 871–887.
24. Baker, C. C., Herisse, J., Courtois, G., Galibert, F. & Ziff, E. (1979) *Cell* **18,** 569–580.
25. Ghosh, P. K., Reddy, V. B., Swinscoe, J., Lebowitz, P. & Weissman, S. M. (1978) *J. Mol. Biol.* **126,** 813–846.
26. Grosschedl, R. & Birnstiel, M. L. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 1432–1436.
27. Osborne, T. F., Gaynore, R. B. & Berk, A. J. (1982) *Cell* **29,** 139–148.
28. Grosveld, G. C., de Boer, E., Shewmaker, C. K. & Flavell, R. A. (1982) *Nature (London)* **295,** 120–126.
29. McKnight, S. L., Gavis, E. R., Kingsbury, R. & Axel, R. (1981) *Cell* **25,** 385–398.
30. Zeitlin, S. & Efstratiadis, A. (1984) *Cell* **39,** 589–602.
31. Birchmeier, C., Schumperli, D., Sconzo, G. & Birnstiel, M. L. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1057–1061.
32. Busch, H., Reddy, R., Rothblum, L. & Choi, Y. C. (1982) *Annu. Rev. Biochem.* **51,** 617–654.
33. Mount, S. M., Pettersson, I., Hinterberger, M., Karmas, A. & Steitz, J. A. (1983) *Cell* **33,** 509–518.
34. Keller, E. B. & Noon, W. A. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 7417–7420.
35. Queen, C. & Korn, L. J. (1984) *Nucleic Acids Res.* **12,** 581–599.
36. Mizuno, T., Chou, M.-Y. & Inouye, M. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1966–1970.
37. Simons, R. W. & Kleckner, N. (1983) *Cell* **34,** 683–691.
38. Izant, J. G. & Weintraub, H. (1984) *Cell* **36,** 1007–1015.