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Transcription of human histone genes in extracts from synchronized HeLa cells

(*in vitro* transcription/cell cycle regulation/stable binding/transcription factor)

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ABSTRACT **Nuclear extracts were prepared from synchronized HeLa cells at various times during the cell cycle and assayed for the ability to transcribe several cellular and viral genes. The efficiency of transcription of a human histone H4 gene is 3- to 10-fold greater in nuclear extracts from S phase nuclei than in extracts from non-S phase cells. In contrast, the adenovirus virus type 2 (Ad2) major late promoter is utilized 3- to 20-fold more efficiently in nuclear extracts from non-S phase cells. Transcription of other genes, including a human histone H3 and the simian virus 40 late transcription unit, is equally efficient in S and non-S phase extracts. Mixing experiments demonstrate that the rate-limiting activities for histone H4 and Ad2 major late transcription function independently and that the effects of these activities are additive. Competition studies suggest that the H4-specific transcription activity can be sequestered by preincubation with the H4 template DNA. These data support the concept that cell cycle regulation of human histone gene transcription may depend in part on soluble transcription activities that are modulated during the cell cycle. Further, in addition to the H4-specific transcription activity, there may exist other transcription factors whose activity can fluctuate according to the cell cycle or according to the growth state of the cells.**

The mechanisms for coupling nuclear DNA synthesis and histone protein production during the eukaryotic cell cycle are not understood. Early investigations in this field showed that translatable histone mRNA is present in significant amounts only during the S phase of the cell cycle and that the inhibition of DNA synthesis results in the rapid and selective loss of histone mRNA from the cell cytoplasm (1–6). More recently, the coupling of histone gene expression and DNA synthesis has been reexamined by using cloned histone genes as hybridization probes to directly measure the contributions of increased transcription rate and mRNA stability to the accumulation of histone mRNA during S phase. It is clear that in synchronized yeast (7, 8) and mammalian cells (9, 10), the increased abundance of histone mRNA during DNA synthesis results both from an increased rate of synthesis and a decreased rate of decay. In particular, our recent study of synchronized HeLa cells indicates that the rate of transcription and the half-life of human histone mRNAs each increase between 3- to 5-fold during S phase (9).

The development of soluble extracts in which cloned eukaryotic genes are accurately transcribed (reviewed in ref. 11) has provided a means for the detailed analysis of those factors involved in eukaryotic mRNA synthesis and processing. One approach towards understanding histone gene regulation during the cell cycle is to reproduce those mechanisms controlling histone mRNA abundance *in vitro*. In this initial report, we describe the preparation of nuclear extracts from synchronized HeLa cells that appear to mimic histone gene regulation *in vitro*. Specifically, we demonstrate that efficient transcription of a cloned human histone H4 gene is dependent on an activity that is present at maximal levels in extracts from S phase HeLa cells and that this activity is not required for transcription of several other eukaryotic genes.

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MATERIALS AND METHODS

Suppliers. Aphidicolin was supplied by the Natural Products Branch, Division of Cancer Treatment, National Cancer Institute. Restriction enzymes were purchased from Bethesda Research Laboratories. Radiolabeled precursors were purchased from New England Nuclear.

Cell Culture and Synchronization. HeLa cells were grown in suspension culture in minimal essential medium (Joklik) supplemented with 5% calf serum. Cell synchronization was by sequential thymidine and aphidicolin blocks, exactly as described in ref. 9.

Extract Preparation and Assay. Nuclear extracts were prepared essentially as described by Dignam *et al.* (12), with the following modifications. Cells were immediately cooled to 4°C prior to centrifugation by pouring over an equal volume of frozen, crushed phosphate-buffered saline. Nuclei were extracted in 2.0 ml of the following buffer per 10^9 cells: 20 mM Hepes, pH 7.9/1.5 mM $MgCl_2$/0.2 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.5 mM dithiothreitol/20% (vol/vol) glycerol/0.60 M KCl. Nuclear extracts prepared in this way contain ≈25 mg of protein per ml and ≈3 mg of nucleic acid per ml. No significant correlations between the protein and nucleic acid concentrations and the stage of the cell cycle from which the extract was prepared were found. Assays were done in a final volume of 10 μl and contained 4–6 μl of extract, 12% glycerol, 0.3 mM dithiothreitol, 12 mM Hepes (pH 7.9), 0.12 mM EDTA, 60 mM KCl, 8 mM $MgCl_2$, 200 μM unlabeled triphosphates (ATP, CTP, UTP), 5 mM creatine phosphate, and 10 μM [^{32}P]GTP (25 Ci/mmol; 1 Ci = 37 GBq). Incubation was at 30°C for 20 min. Gel electrophoresis, processing, and autoradiography were as described (12). Densitometry was done by using a Beckman Du8 spectrophotometer equipped with a gel-scanning accessory. Multiple exposures were processed and scanned to ensure accuracy.

RESULTS

Transcription Templates. The human histone subclones employed in this study are designated pHu4A and pHh5B and contain a human histone H4 and H3 gene, respectively. We have previously reported the complete nucleotide sequence, the positions of the 5′ and 3′ mRNA termini, and changes in the steady-state concentration of these mRNAs during the HeLa cell cycle (13). Plasmids containing the adenovirus major late promoter (pSmaF) and the simian virus 40 (SV40) late promoter (Y182) have been routinely utilized for *in vitro* transcription studies in this laboratory and are de-

Abbreviations: Ad2, adenovirus type 2; SV40, simian virus 40.

Table 1. *In vitro* transcription templates

Promoter transcript	Name	Ref.	Restriction cleavage	Expected runoff
Human histone H4	pHu4A(H4)	13	*Hin*dIII	620
			*Bam*HI	950
Human histone H3	pHh5B(H3)	13	*Xba* I	1200
Adenovirus major late	pSmaF(Ad2)	14	*Sma* I	536
			*Hin*dIII	194
SV40 late	Y182(SV40)	15	*Eco*RV	630

Ad2, adenovirus type 2.

scribed elsewhere (14–16). Table 1 provides a complete listing of the templates employed in this study and the size of expected runoff transcription products resulting from cleavage with relevant restriction endonucleases.

Human Histone Gene Transcription *in Vitro*. In our initial analysis, transcription of the pHu4A histone H4 template was studied in nuclear extracts prepared from unsychronized HeLa cells. Two important results were obtained: first, transcription of this H4 gene initiates at or very close to the *in vivo* initiation site; second, the relative efficiency of transcription from the H4 and Ad2 promoters can vary significantly (data not shown). In fact, although the relative efficiencies of transcription from these promoters is constant in fresh aliquots of a given extract, transcription of the H4 template is between 5% and 40% as efficient as the adenovirus template in different nuclear extracts from unsynchronized cells. These results provided the first indication that the rate-limiting events for transcription of these two promoters *in vitro* may be distinct. Further support for this idea comes from the fact that some preparations of highly purified fractions that reconstitute major late transcription very efficiently are not able to transcribe the H4 template (unpublished work).

Studies of RNA synthesis *in vivo* indicate that the rate of histone gene transcription specifically increases between 3- and 5-fold during S phase (9, 10). We wished to determine whether histone gene transcription *in vitro* is dependent upon a soluble component whose activity is modulated during the cell cycle. Hence, nuclear extracts were prepared from synchronized cells at various points during the traverse of the cell cycle. Typically, 2×10^9 HeLa cells were synchronized by successive thymidine and aphidicolin blocks and divided into equal aliquots, and extracts were prepared at 2.5 hr after release into S phase and at 10–12 hr after release. Our previous *in vivo* measurements indicated that histone mRNA is transcribed at maximal rates early in S phase (i.e., 2.5 hr after release) and at minimal rates either prior to entry into S phase (before release from the block) or following S phase (10–15 hr after release) (9). Fig. 1 shows transcription of the H4 and Ad2 templates in two pairs of cell cycle extracts.

The relative efficiency of transcription from the H4 gene and the Ad2 template change dramatically in extracts prepared from S phase (Fig. 1, lanes 1 and 3) and non-S phase HeLa cells (Fig. 1, lanes 2 and 4). Specifically, transcription of the histone template is 3- to 10-fold more active in the S phase extracts than in the corresponding non-S phase extracts, whereas the adenovirus template is transcribed 3- to 20-fold better in the non-S phase extracts than in the S phase extracts. The combined effect of these differences in transcription efficiency are most evident if one compares the transcription of the H4 and Ad2 template DNAs in the non-S phase nuclear extracts (Fig. 1, lanes 2 and 4, respectively). In this case, the Ad2 template DNA is transcribed between 10- and 50-fold more efficiently than the H4 template. Three conditions seem particularly important for consistent reproduction of these results: the cell populations must be very well synchronized; time of preparation of the extracts should be minimal; and the nuclear extract should not be frozen and thawed repeatedly prior to use.

The observed differences in H4 and Ad2 transcription in nuclear extracts from S and non-S phase cells do not result from differences in the stability of the *in vitro* transcripts, the time at which the accumulation of the H4 and Ad2 RNA products plateau, or changes in the nucleotide or DNA optima for transcription (data not shown). Supplementation of the nuclear extract with the cytoplasmic fraction from the same cells had no consistent effect on the transcription efficiency of either template DNA. Finally, several other cellular and viral DNA templates, including the pHh5B human histone H3 gene and the SV40 late transcription unit (see below), have been assayed in these extracts and reveal no changes in their transcription efficiency. It appears, therefore, that the rate-limiting components for *in vitro* transcription of this histone H4 gene and the adenovirus major late promoter may be distinct.

To investigate this further, we performed a series of experiments in which the ratio of S phase to non-S phase extract in the assay was systematically varied, while the total extract concentration remained unchanged. As shown in Figs. 2*A* and 3*A*, the transcription efficiency of the histone H4 template is directly dependent on the amount of S phase extract in the assay. In contrast, transcription of the Ad2 major late promoter is proportional to the amount of non-S phase extract (Figs. 2*B* and 3*B*). It is noteworthy that in the case of the histone H4 template, transcription of vector sequences (as evidenced by the intensity of the long transcripts at the top of each lane in Fig. 2*A*) is not proportional to transcription of the H4 gene but, rather, is correlated with the

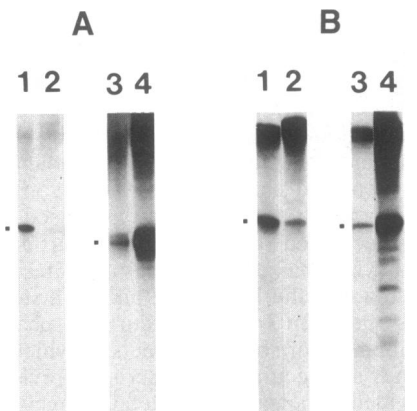

FIG. 1. Transcription of the histone H4 (pHu4A) and Ad2 major late (pSmaF) promoters in extracts from synchronized HeLa cells. (*A* and *B*) Analogous results obtained with cell cycle extracts prepared on separate days. Lanes 1, transcription of *Hin*dIII-cleaved pHu4A DNA template in S phase nuclear extract; lanes 2, transcription of pHu4A template in non-S phase nuclear extract; lanes 3, transcription of *Sma* I-cleaved pSmaF template DNA in S phase nuclear extract; lanes 4, transcription of pSmaF template DNA in non-S phase nuclear extract. ■, Expected runoff transcription product.

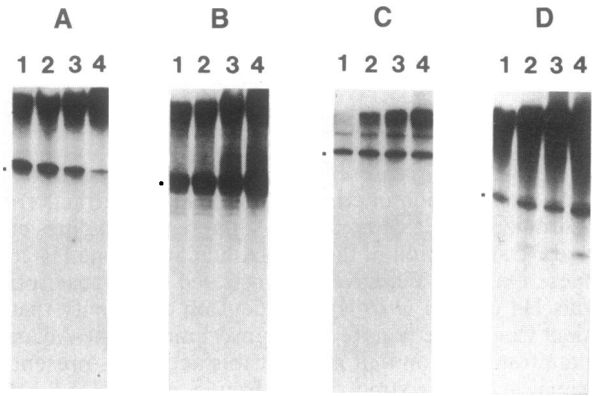

FIG. 2. Effect of mixing S and non-S phase nuclear extracts on *in vitro* transcription. In each reaction, the following amounts of S and non-S phase extracts were used: lanes 1, 6.0 μl of S extract; lanes 2, 4.0 μl of S and 2.0 μl of non-S extract; lanes 3, 2.0 μl of S and 4.0 μl of non-S extract; lanes 4, 6.0 μl of non-S extract. DNA templates employed were *Hin*dIII-cleaved pHu4A (human histone H4) (*A*), *Sma* I-cleaved pSmaF (adenovirus major late) (*B*), XbaI-cleaved pHh5B (human histone H3) (*C*), and *Eco*RV-cut Y182 (SV40 late) (*D*).

amount of non-S phase extract present in the reaction. Finally, for both the human histone H3 gene and the SV40 late transcription unit the efficiency of transcription does not change appreciably with the changing ratios of S and non-S phase nuclear extract (Figs. 2*C* and 3*A*, Figs. 2*D* and 3*B*).

From these data we conclude that the rate-limiting factors for H4 and Ad2 transcription are distinct and function independently, even when present in the same assay. To determine whether the non-S phase extract was contributing significantly to the H4 transcription in these experiments and the extent to which the S phase extract influenced Ad2 transcription, simple dilution experiments were performed. For comparative purposes, the results of the buffer dilution experiments are plotted on the same axis as the mixing experiment described above (Fig. 3). Mixing the S extract with the non-S extract or diluting it with buffer resulted in a similar reduction in the transcription of the H4 template DNA (Fig. 3*A*). In an analogous experiment, Ad2 transcription was decreased to a similar extent by mixing of the non-S phase extract with the S phase extract or by dilution with buffer (Fig. 3*B*). These results are particularly relevant in considering H4 transcription, since they indicate that the relative abundance of those transcription factors required for Ad2 transcription in the non-S phase extract have very little effect on the transcription of the H4 template. Rather, the majority of the rate-limiting component for histone H4 transcription is present in the S-phase nuclear extract.

Competition Studies. As an independent test for the presence of a histone H4-specific transcription factor and to ask whether this activity exerts its effect through specific interaction with the template DNA, competition assays were employed. This experimental approach is very closely modeled after previous studies by Bogenhagen *et al.* (17) and Lassar *et al.* (18) to determine the requirements for stable complex formation on several RNA polymerase III templates *in vitro*. The assay is designed to detect a transcription factor by its ability to be specifically sequestered by a given DNA template during preincubation with that template in the absence of detectable transcription. Several preliminary experiments were done to identify and characterize an appropriate S phase nuclear extract for these studies. The extract chosen was completely dependent on exogenously added nucleotide triphosphates for detectable transcription and transcribed the H4 template ≈50% as efficiently as it transcribed the Ad2 template. This last condition (i.e., an excess of Ad2 transcription components over the H4 specific activity) was chosen for the following reason. If transcription of these templates requires any common component, as is almost certainly the case, then it is desirable to provide an excess of that component in the preincubation so that titration of the H4-specific activity is reached prior to titration of the shared component. In this way, sufficient transcription activity remains after the preincubation step to allow for assay of the secondary DNA templates.

In the experiment shown in Fig. 4*A* increasing amounts of the histone H4 template cut with *Bam*HI (generating a 950-nucleotide runoff transcription product) were preincubated with the nuclear extract in the absence of nucleotide triphosphates. After 10 min, a mixture of the H4 template cut with *Hin*dIII (generating a 620-nucleotide runoff transcription product) and the Ad2 template were added along with nucleoside triphosphates, and the incubation was continued for an additional 20 min. The DNA concentrations of the secondary DNA templates were adjusted to produce nearly equivalent signals during the transcription reaction. As expected, as the concentration of the *Bam*HI-cut histone H4 template is increased in the preincubation step, the accumulation of the 950-base-pair runoff transcript increases. On the other hand, transcription of the secondary H4 template DNA decreases markedly relative to the Ad2 template DNA. These data are presented quantitatively in Fig. 5*A* (dashed lines). Identical results were obtained when an isolated DNA fragment carrying the H4 gene free of vector sequences was used in the

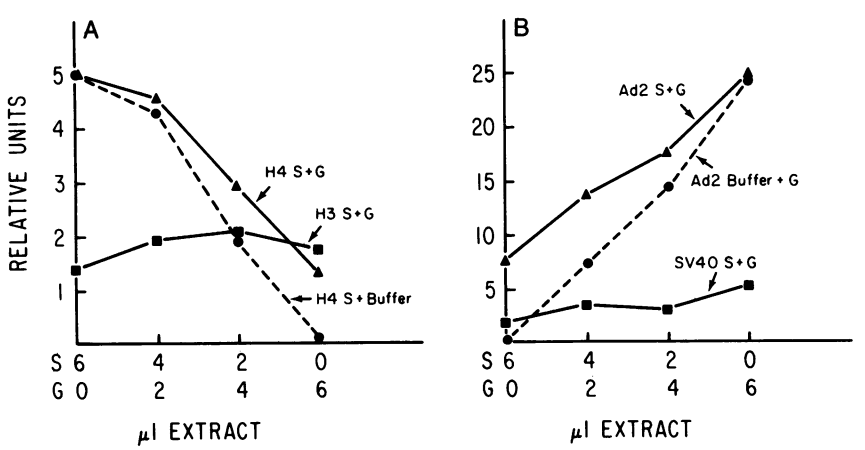

FIG. 3. Quantitation of mixing experiment shown in Fig. 2 (solid lines). Also plotted are results of dilution experiments in which S extract was diluted with buffer and assayed for H4 transcription (*A*, ●-----●) or G1 extract was diluted with buffer and assayed for Ad2 transcription (*B*, ●-----●).

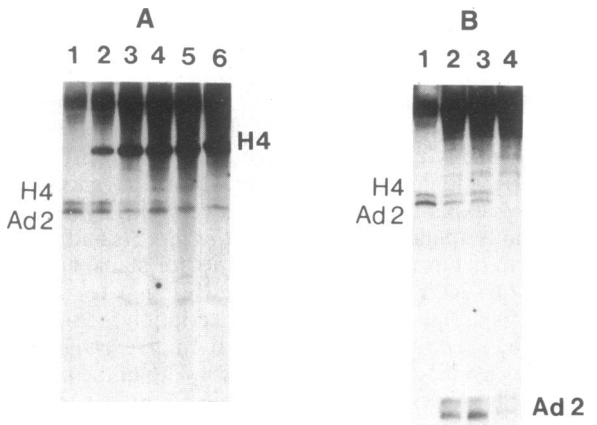

FIG. 4. Ability of H4-specific transcription activity to be sequestered by H4 template DNA. (*A*) Increasing quantities of a primary H4 template DNA (*Bam*HI-cleaved pHu4A; bold characters) were incubated with the nuclear extract for 10 min; NTPs, 250 ng of secondary H4 template (*Hin*dIII-cleaved pHu4A) and 100 μg of Ad2 template (*Sma* I-cleaved pSmaF) were added and transcription was allowed to occur for 20 min. Lanes indicate reactions in which the following amounts of H4 primary DNA template were included into the first incubation: lane 1, no DNA; lane 2, 200 ng; lane 3, 400 ng; lane 4, 600 ng; lane 5, 800 ng; lane 6, 1000 ng. (*B*) Analogous experiment in which the following quantities of a primary Ad2 major late primary DNA template (*Hin*dIII-cleaved pSmaF) were included in the primary incubation: lane 1, no DNA; lane 2, 250 ng; lane 3, 500 ng; lane 4, 1000 ng.

primary incubation (data not shown). In some experiments (Fig. 5*A*, solid lines), the decrease in transcription of the secondary H4 template was accompanied by an increase in transcription of the secondary Ad2 DNA. This is of interest since it may reflect competition for a common transcription component, which occurs only when the H4 template is complexed with its specific transcription factor.

In contrast, when the Ad2 template was included in the primary incubation instead of the H4 template DNA (Figs. 4*B* and 5*B*), qualitatively different results were obtained. In this case, as transcription from the primary Ad2 template increases, accumulation of transcripts from both secondary template DNAs decrease at the same rate. Similar results were obtained by using pBR325 DNA in the primary incubation (data not shown). These data further support the suggestion that H4 and Ad2 transcription require a common component.

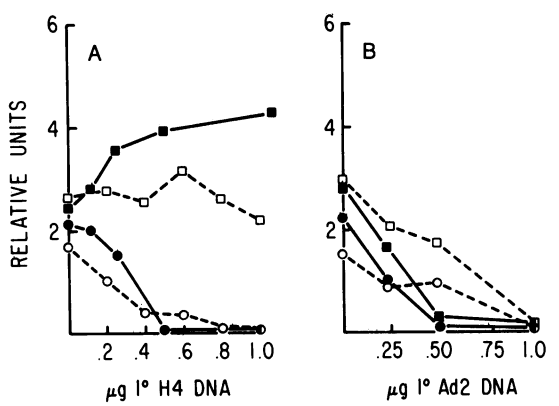

FIG. 5. Quantitation of competition experiment shown in Fig. 4 (open symbols). Closed symbols show values obtained for independent experiment performed in exactly the same manner. (*A*) Primary template, *Bam*HI-cleaved pHu4A; ○ and ●, Transcription of secondary H4 template; □ and ■, transcription of secondary Ad2 template. (*B*) Primary template, *Hin*dIII-cleaved pSmaF; ○ and ●, Transcription of secondary H4 template; □ and ■, transcription of secondary Ad2 template.

DISCUSSION

The present study represents our initial characterization of *in vitro* transcription in crude soluble nuclear extracts from synchronized HeLa cells. Our most interesting findings concern the transcription of the pHu4A human histone H4 gene in these extracts. We have demonstrated that transcription of this H4 gene *in vitro* is dependent on an activity that is distinct from those required for transcription of the Ad2 major late transcription unit and that this activity is present at maximal levels in extracts made from S-phase HeLa cells. The activity is rate limiting for histone H4 transcription *in vitro*, even when mixed with extracts that are very active for Ad2 late transcription. Furthermore, we have shown that a component(s) required for histone H4 transcription can be specifically sequestered during preincubation with either the entire pHu4A plasmid DNA or the isolated insert from this plasmid. It is most probable that the rate-limiting activity in the S phase nuclear extract and the component sequestered during preincubation with the H4 template are identical. In this case, the simplest interpretation of this data is that transcription of the histone H4 gene *in vitro* requires a component that interacts with the DNA template and whose activity is most abundant during S phase. These properties are consistent with our expectations of a protein that may be involved in regulating histone gene expression *in vivo*. If this H4-specific activity is, in fact, involved in histone gene regulation *in vivo*, then the observation that *in vitro* transcription of the histone H3 gene is not affected by this activity suggests that there may be separate factors for modulating the transcriptional activity of the various histone gene subtypes (i.e., H1, H2a, H2b, H3, and H4). Alternatively, those sequences necessary for cell cycle-regulated transcription may not be present on the pHh5B subclone.

An equally intriguing result is that *in vitro* transcription of the adenovirus major late promoter fluctuates so dramatically in the cell cycle extracts. This raises the exciting possibility that the expression of additional cellular and viral genes may be modulated by a general transcription component whose activity changes during the cell cycle or according to the growth state of the cell. Obviously, further examination of this possibility is accessible through the *in vitro* approach utilized in this study. Furthermore, consideration of our results with the recent discoveries of SV40 (19) and *Drosophila* heat shock (C. S. Parker, personal communication) gene-specific transcription factors results in an appreciation for the multiplicity of proteins controlling specific gene transcription *in vitro* and, probably, *in vivo*.

At this point, it is premature to discuss at length the implications these results have for models of histone gene regulation *in vivo*. It seems likely, however, that histone gene transcription *in vivo* may be controlled both by changes in the structure of the template DNA during the cell cycle (9) and by modulation of the activity of histone gene-specific transcription factors. Obviously, a detailed knowledge of both the mechanism of action of the H4-specific transcription activity and of the nucleotide sequences necessary for its activity *in vitro* compared with sequences required for cell cycle regulation *in vivo* must be obtained.

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1. Robbins, E. & Borun, T. W. (1967) *Proc. Natl. Acad. Sci. USA* **58,** 1977–1983.
2. Butler, W. B. & Meuller, G. C. (1973) *Biochim. Biophys. Acta* **294,** 481–496.
3. Borun, T. W., Gabrielli, K., Asiro, Zwiedler, A. & Baglioni, C. (1975) *Cell* **4,** 59–67.
4. Briendl, M. & Gallwitz, D. (1974) *Eur. J. Biochem.* **45,** 91–97.
5. Gallwitz, D. (1975) *Nature (London)* **257,** 247–248.
6. Detke, S., Lichtler, I., Philips, I., Stein, J. & Stein, G. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4995–4999.
7. Hereford, L., Osley, M. A., Ludwig, J. R. & McLaughlin, C. S. (1981) *Cell* **24,** 367–375.
8. Hereford, L., Bromley, S. & Osley, M. A. (1982) *Cell* **30,** 305–310.
9. Heintz, N., Sive, H. L. & Roeder, R. G. (1983) *Mol. Cell. Biol.* **3,** 539–550.
10. Sittman, D. B., Graves, R. B. & Marzluff, W. F. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 1849–1853.
11. Heintz, N. & Roeder, R. G. (1982) in *Genetic Engineering*, eds. Setlow, J. K. & Hollaender, A. (Plenum, New York), Vol. 4, pp. 57–89.
12. Dignam, J. D., Lebowitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
13. Zhong, R., Roeder, R. G. & Heintz, N. (1983) *Nucleic Acids Res.* **11,** 7409–7425.
14. Weil, P. A., Luse, D. S., Segall, J. & Roeder, R. G. (1979) *Cell* **18,** 469–484.
15. Hamer, D. H. (1980) in *Genetic Engineering*, eds. Setlow, J. K. & Hollaender, A. (Plenum, New York), Vol. 2, pp. 88–101.
16. Luse, D. S. & Roeder, R. G. (1980) *Cell* **20,** 691–699.
17. Bogenhagen, D. F., Wormington, W. M. & Brown, D. D. (1982) *Cell* **28,** 413–421.
18. Lassar, A. B., Martin, P. & Roeder, R. G. (1983) *Science* **222,** 740–748.
19. Dynans, W. S. & Tjian, R. (1983) *Cell* **32,** 669–680.