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OVEC, a versatile system to study transcription in mammalian cells and cell-free extracts

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ABSTRACT

We have developed a vector, OVEC ("oligonucleotide vector") to study DNA sequences involved in the regulation of transcription in mammalian cells. This vector is equally suitable for studying expression in vivo after transfection into cells, or for transcription studies in vitro with cell-free extracts. Putative cis-acting DNA segments from enhancers or promoters can be inserted at a position immediately upstream of the TATA box and coding sequence of the rabbit β-globin gene. A regulatory DNA segment can be tested by itself or in conjunction with an enhancer located either in an adjacent upstream position, or downstream of the β-globin gene. S1 nuclease mapping can be used to study transcription from circular and linear templates and run-off transcription in vitro is also feasible. Transcripts from a reference globin gene with a small deletion around the transcription initiation site can be measured with the same S1 nuclease probe and thus serve as an internal standard. We demonstrate the usefulness of OVEC by inserting either short oligonucleotides comprising a metal responsive enhancer element, or the SV40 enhancer, directly upstream of the TATA box. Both constructs yield high levels of correctly initiated transcripts in a transient expression assay in HeLa cells. In a HeLa cell nuclear extract the SV40 enhancer stimulates transcription 40-fold.

INTRODUCTION

A very important aspect of molecular biology is the study of transcriptional regulation of cloned genes after their reintroduction into living cells. However, experiments to compare activities of regulatory sequences in different cell types can be hampered by the possibility of differential transcript stability. Therefore, it has become common to link cis-acting regulatory sequences to a so-called reporter gene. Several transcription assay systems have been described for studies in vivo (1-5) and in vitro (6,7 and references therein). In some cases attempts have been made to measure the transcription

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activity in vivo and in vitro in parallel with the same gene constructs (8,9). Usually, however, the in vivo test systems are not used for in vitro transcription and vice versa because of technical difficulties (e.g. no splicing or enzyme production in vitro; transcript instability in vivo). In this paper we describe a vector (OVEC) which combines the advantages of test gene systems for both in vivo and in vitro expression. In particular, the same internal control is used for in vivo and in vitro assays. This system is therefore ideally suited to extend a transcriptional analysis in vivo to experiments with cell-free extracts. Such in vitro systems are instrumental for an understanding of transcription regulation at the molecular level.

Although transcription control elements are usually grouped into two classes, promoters and enhancers, these classes can overlap both physically and functionally. For example, most enhancer elements studied so far are also found to stimulate transcription when inserted upstream of a TATA box (although not every promoter element can enhance transcription over long distances; G. Westin, unpublished results). Enhancers/promoters of mammalian genes have a modular structure and are composed of an array of sequence motifs (10). In principle, enhancer motifs can act independently, but each additional element generally yields an incremental increase in transcriptional activity (11; G. Schaffner et al., unpublished results). Many, and perhaps all of these sequence motifs are binding sites for transcription factors. In some cases, binding sites for a given transcription factor occur both in the promoter and the enhancer regions. This is particularly striking in case of a decanucleotide motif which is present both in the immunoglobulin heavy chain enhancer located in the first intron, and also in immunoglobulin variable gene promoters (12,13).

It is known that the TATA box alone is not sufficient for full response to a remote enhancer (14,15). Addition of one or two cis-acting DNA motifs upstream of the TATA box can convert this region into a promoter that may be weak by itself but will respond strongly to a remote enhancer positioned, for example, downstream of a test gene. Addition of several further sequence motifs upstream of the TATA box, however, results in the build-

up of an enhancer-promoter region that does not require the presence of a remote enhancer. Finally, a negatively acting DNA segment can also be investigated which acts when placed between an enhancer and the TATA box. Such a silencer was recently identified in the human IFN-alpha promoter by C. Weissmann and his colleagues (16, see also 17,18 for β-IFN).

MATERIALS AND METHODS

All enzymatic manipulations were done according to standard procedures (19). The Gene Assembler (Pharmacia) was used for the oligonucleotide synthesis.

Construction of OVEC

The OVEC plasmid was made in three steps: (i) the SacI/Asp718 fragment containing the rabbit β-globin gene (extending between positions -1250 and +3300), with an XhoI site at position -425, was ligated to SacI/SphI digested pUC18 plasmid (20). The pUC18 vector was first modified so that the EcoRI site in the polylinker sequence was converted into an additional HindIII site. After ligation at the SacI site, the incompatible Asp718 and SphI ends were blunt-ended by the use of T4 DNA polymerase (after ligation this procedure recreates the Asp718(KpnI) site). After a second ligation, E.coli HB101 cells were transformed and a clone was selected for further manipulation. (ii) The SacI site in this plasmid was destroyed by SacI digestion followed by incubation with T4 DNA polymerase and religation. (iii) The XhoI/BamHI fragment extending between positions -425 and +475 was then replaced by two other fragments. Firstly, a double-stranded synthetic oligonucleotide comprising β-globin gene sequences from position -37 to the PvuII site at position -10 and a SacI and a SalI site immediately upstream of position -37. Two base pair changes were introduced to create a PstI site overlapping the PvuII site. The sequence of this oligonucleotide is:

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5'TCGAAGAGCTCACTGTGTCGACCTTGGGCATAAAAGGCAGAGCACTGCAG 3'
    3'TCTCGAGTGACACAGCTGGAACCCGTATTTTCCGTCTCGTGACGTC 5'
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(the SacI, SalI and PstI sites are underlined).
Secondly, a PvuII/BamHI fragment extending between positions -10

and +475, but derived from a clone lacking the first intervening sequence (21), was also inserted.

Construction of OVEC-REF

Instead of using the PvuII/BamHI fragment as described above a PvuII/BamHI fragment extending between positions +19 and +475 was used in the ligation reaction. This fragment was derived from a globin gene with a point mutation at position +19 which creates a PvuII cleavage site (22). A 196 bp fragment containing the SV40 enhancer (23) was subsequently cloned into a blunt-ended SalI site upstream of the globin gene.

Construction of SV-OVEC

The 196 bp long fragment with the SV40 enhancer used above was cloned into a blunt-ended SalI site in the OVEC vector.

Construction of MRE(4x)-OVEC

A double-stranded oligonucleotide was synthesized with the general structure of a model oligonucleotide as shown in Fig.1B, containing four tandem copies of an 18 bp segment comprising a metal responsive element (MRE, from the mouse metallothionein-I gene; 24,25). The sequence of the oligonucleotide is

5'CGAGGAGCTCTGCACTCCGCCCGAGCTCTGCACTCCGCCCGAGCTCTGCACTCCGCC
3'TCGAGCTCCTCGAGACGTGAGGCGGGCTCGAGACGTGAGGCGGGCTCGAGACGTGAGGCGG

CGAGCTCTGCACTCCGCCCG 3'
GCTCGAGACGTGAGGCGGGCAGCT 5'

(the XhoI site is underlined). The MRE oligonucleotide was cloned into SacI/SalI digested OVEC and the DNA sequence was determined according to Maxam and Gilbert (26).

Construction of β1E-SV

The 196 bp EcoRI fragment with the SV40 enhancer was inserted into the EcoRI site of pβ1E. This plasmid is identical to pβG (23) except that the EcoRI site in the third exon is removed, thereby leaving a unique EcoRI site downstream of the gene (27).

DNA transfection and RNA analysis

HeLa cells were grown in DMEM supplemented with 2.5% fetal calf serum, 2.5% calf serum, 100U/ml penicillin and 100U/ml streptomycin. The cells were transfected by calcium phosphate co-precipitation (21,28). 20μg test gene plasmid and 1μg OVEC-REF were used per 100 mm plate. 8-12 hours after

transfection the cells were shocked for 3 min with 25% DMSO in Tris-buffered saline (TBS) and then washed twice with TBS and incubated with fresh medium (21). In the metal ion induction experiment 10^{-4} M $ZnSO_4$ and 0.5 x 10^{-6} M $CdSO_4$ were added to the medium one hour after the DMSO shock and after 8-12 hours the medium was changed and the metal ion concentration was increased to 2 x 10^{-4} M $ZnSO_4$ and 10^{-6}M $CdSO_4$ (29). Cytoplasmic RNA was isolated 40-44 hrs after transfection and residual input plasmid DNA was removed by treatment with RNase-free DNase (21,30). S1 nuclease analysis (1,21) was performed with a single-stranded oligonucleotide probe extending between positions -18 and +75 on the non-coding strand in the rabbit β-globin gene (the S1 probe sequence is 3' CGTCCCGTCGACGACGAATGTGAACGAAAACTGTGTTGACACAAA TGAACGTTAGGGGGTTTTGTCTGTCTTACCACGTAGACAGGTCACTCC 5'. Samples were analyzed in a 10% polyacrylamide (19:1) 7.5M urea gel.

Preparation of nuclear extracts and in vitro transcription

Suspension cultures of HeLa cells were grown to a density of approximately 5 x 10^5 cell/ml in Joklik's minimal essential medium supplemented with 5% calf serum. Nuclear extracts were prepared as described by Dignam et al. (31), with minor modifications. After $(NH_4)_2SO_4$ precipitation (0.33g/ml), the extract was resuspended in and dialyzed against 20mM HEPES-KOH pH7.9, 20% glycerol, 20mM KCl, 2mM $MgCl_2$, 0.2mM EDTA and 0.5mM dithiothreitol. In vitro transcription reactions (10µl) contained 2µl HeLa nuclear extract, 0.4µg test gene and 0.2µg OVEC-REF (when indicated), 10mM HEPES-KOH pH7.9, 10% glycerol, 20mM KCl, 4mM $MgCl_2$, 4mM spermidine, 0.1mM EDTA, 0.25mM dithiothreitol, 5mM creatine phosphate, 0.5mM ATP, 0.5mM GTP, 0.5mM UTP and 0.5mM CTP. The samples were incubated at 30°C for 60 min and 10µl of 8M urea, 2% SDS and 20mM EDTA was added. The samples were boiled for 3 minutes prior to phenol/chloroform/ isoamylalcohol (25:24:1) extraction and ethanol precipitation. The RNA was analyzed by S1 nuclease mapping and polyacrylamide gel electrophoresis as described above.

RESULTS

We have developed a versatile test vector, OVEC ("oligo-nucleotide vector") to study enhancer/promoter elements involved

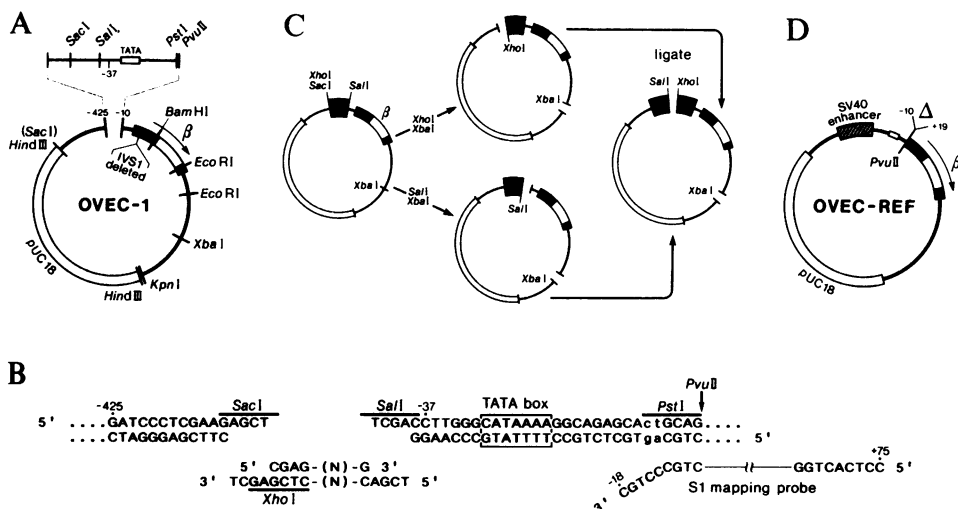

Fig.1: A) Map of OVEC. This vector is based on a rabbit ß-globin gene whose first intervening sequence (IVS1) and upstream sequences between positions -425 and -37 are deleted. A SacI and a SalI site have been introduced immediately upstream of the ß-globin gene TATA box (the "ATA" box, 14) and a PstI site overlapps the natural PvuII site at position -10.
B) The nucleotide sequence covering part of the promoter region in OVEC digested with SacI and SalI. Lower case letters indicate mutations introduced to create the PstI site. Shown below is a model oligonucleotide with SacI and SalI sticky ends and extra nucleotides comprising an XhoI site. Also indicated is part of the 93 nt S1 nuclease mapping probe which contains five non-complementary nucleotides at the 3'end. This allows non-digested probe and readthrough transcripts starting further upstream to be distinguished.
C) Creation of multiple copies of a cloned oligonucleotide. Multimerization to two copies is demonstrated, further copies can be obtained by repeating the procedure. Identical or different sequence motifs can be assembled in this way.
D) Map of OVEC-REF. This reference gene contains a 28 bp deletion around the transcription initiation site. The SV40 enhancer is inserted at the SalI site.

in transcriptional regulation. The rabbit β-globin gene was chosen as a test gene since it yields extremely stable transcripts in a great variety of cells (2,11,32-34). In transient expression assays, we have used it mostly in HeLa cells but also in several cell lines from different tissues or species (34; M. Pettersson and W. Schaffner, unpublished results).

The general structure of OVEC is outlined in Figure 1A and a

detailed description of the different plasmids used in this study is presented in the Materials and Methods section. We have designed the vector such that it can be opened by SacI and SalI digestion upstream of the β-globin TATA box (the "ATA" box; 14) thus creating a 3' and a 5' overhang. DNA segments, notably synthetic double-stranded oligonucleotides comprising a single- or tandemly repeated enhancer/promoter motif(s) can be cloned between these two sites in a predetermined orientation (see Fig.1B). In principle, the combination of a 3' and 5' overhang should also be useful for directly cloning single-stranded DNA segments (35,36). In those cases where a DNA sequence motif is tested by itself without further enhancer sequences, multiple copies may be required for efficient transcription. Such recombinants could be easily constructed as shown in Fig.1C, if an XhoI site is built into the oligonucleotide. In addition, a PstI site was generated at position -10, between the TATA box and the initiation site. In this way, the globin TATA box can be replaced if necessary by a heterologous TATA box.

We have synthesized a short, single stranded oligonucleotide as an S1-nuclease probe which eliminates the need for strand separation. The probe, of 93 nucleotides starting at position -18, allows the identification of correctly initiated transcripts that hybridize to the end-labelled oligonucleotide over a length of 75 nucleotides (Fig. 1B). Transcripts derived from a reference gene can be mapped with the same oligonucleotide. This reference gene (OVEC-REF) has a deletion of 28 bp around the transcription initiation site and is driven by an SV40 enhancer in the SalI site upstream of the TATA box (Fig. 1D). Due to the selection of a new initiation site an S1 resistant DNA fragment of 47 nt is generated with reference gene transcripts (see below). The use of an oligonucleotide probe also has the advantage that enough material for thousands of S1 mapping experiments can be prepared in one DNA synthesis, and that samples can be end-labelled to a high specific activity. The fact that the oligonucleotide probe contains five non-complementary nucleotides at the 3' end allows non-digested probe and readthrough transcripts starting further upstream to be distinguished (see below).

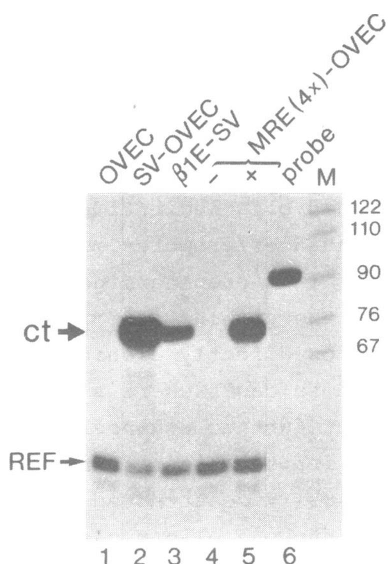

Fig.2: Transcription in vivo. Quantitative S1 nuclease mapping of β-globin RNA isolated from HeLa cells transfected with 1µg OVEC-REF plasmid and 20 µg of the following plasmids. OVEC, without enhancer (lane1); SV-OVEC, containing the SV40 enhancer immediately upstream of the TATA box (lane2); β1E-SV, containing the SV40 enhancer in a downstream position (lane3). The MRE(4x)-OVEC plasmid was transfected to duplicate plates, and RNA from non-induced cells (-) and from metal ion-induced cells (+) was mapped (lanes 4 and 5). A 93 nt S1 probe (Fig.1B) was used (lane6). M, HpaII-digested pBR322 marker DNA; ct, correctly initiated transcripts; REF, reference gene transcripts.

To demonstrate the usefulness of OVEC, we have constructed two test gene plasmids, namely with (i) the SV40 enhancer inserted immediately upstream of the TATA box (SV-OVEC), and (ii) four tandemly repeated copies of an 18 bp oligonucleotide comprising a metal ion-inducible enhancer from the mouse metallothionein-I gene (MRE(4x)-OVEC). The OVEC, SV-OVEC and MRE(4x)-OVEC plasmids were transfected together with the reference gene OVEC-REF into HeLa cells using the calcium phosphate method (21,28). Cytoplasmic RNA was prepared 40-44 hours later and analyzed by S1-nuclease mapping using the labelled single stranded 93 nt oligonucleotide as probe. The results presented in Figure 2 show that in the vector with the SV40 enhancer abutting the TATA box (lane 2, SV-OVEC)

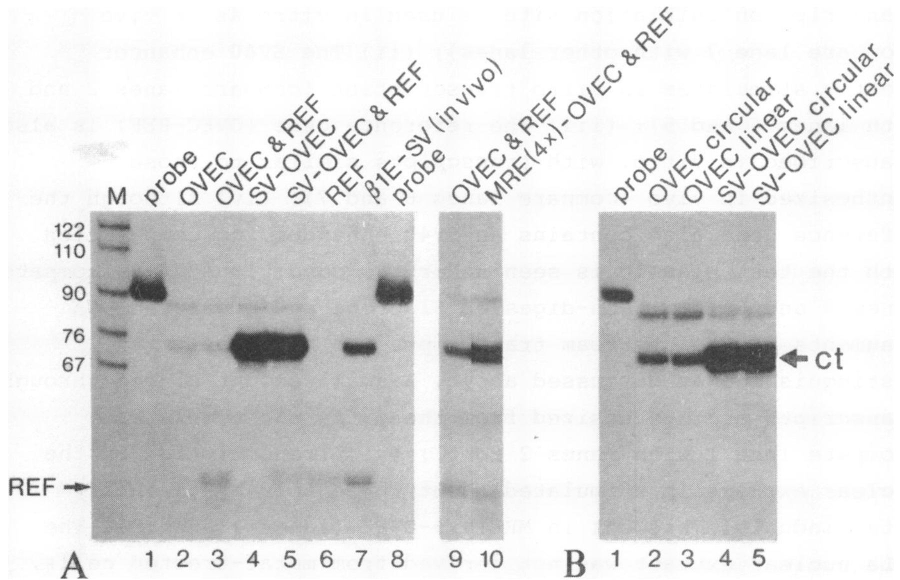

Fig.3: Transcription in vitro. A) Quantitative S1 nuclease mapping of ß-globin RNA synthesized from circular templates in a HeLa cell nuclear extract. The templates are described in the legend to Fig.2. The reference gene (OVEC-REF) is denoted REF. B) S1 nuclease mapping of RNA synthesized from circular and linear templates.

transcription is dramatically stimulated over the level seen with no enhancer (lane 1, OVEC) and also compared to a construct with the complete globin gene promoter where the SV40 enhancer is in a downstream position (lane 3, β1E-SV). The plasmid containing a heavy metal-inducible enhancer (MRE(4x)-OVEC) shows a high level of transcription in metal ion-induced cells (+) as compared to non-induced cells (-) (lanes 4 and 5). Transcripts from the reference gene OVEC-REF are slightly heterogeneous at their 5' end, probably because the natural site for transcription initiation has been deleted. However, the relative intensity of the REF transcript bands was found to be highly reproducible.

The OVEC constructs were also tested in a nuclear extract prepared from HeLa cells. Figure 3A shows S1-nuclease mapping of RNA synthesized from circular templates in the HeLa cell extract. The results are summarized as follows: (i) The same

transcription initiation site is used in vitro as in vivo (compare lane 7 with other lanes); (ii) The SV40 enhancer greatly stimulates in vitro transcription (compare lanes 2 and 3 with lanes 4 and 5); (iii) The reference gene (OVEC-REF) is also transcribed in vitro, with transcripts similar to those synthesized in vivo (compare lanes 6 and 7); (iv) Although the reference gene also contains an SV40 enhancer, no competition with the test plasmid is seen under the conditions used (compare lanes 5 and 6); (v) Non-digested S1 probe and protected DNA fragments due to upstream transcripts can be clearly distinguished, as discussed above. A small amount of readthrough transcripts are synthesized from the different templates (compare lane 1 with lanes 2 to 6); (vi) Transcription in the nuclear extract is stimulated about three-fold by the heavy metal inducible element in MRE(4x)-OVEC (lanes 9 and 10). The HeLa nuclear extract was not derived from metal-treated cells, nor was there any addition of heavy metal to the transcription reaction. Therefore, the weak stimulation may represent the basal activity of this enhancer also seen in vivo.

Using different in vivo and in vitro transcription assays, various groups have obtained conflicting evidence about the requirement for supercoiled template DNA (8,9,37,38,39). For this reason we compared the transcriptional activity of linear and circular templates with and without the SV40 enhancer. The template DNA was linearized within the test gene at the BamHI site. This allows direct RNA mapping by run-off transcription but prevents RNA polymerase II from entering at a free DNA end close to the enhancer/promoter region. The S1-nuclease analysis presented in Fig.3B shows that the topology of the template does not significantly affect transcription in HeLa cell nuclear extracts. Therefore, it seems unlikely that the stimulatory effect of the SV40 enhancer on transcription in vitro depends on DNA topology.

DISCUSSION

We have constructed OVEC to study transcription both in mammalian cells in vivo and in cell-free extracts. The possibility of studying cis-acting regulatory elements of transcription in parallel in both systems, with the same

constructions and with the same probe, should allow us to systematically extend previous in vivo studies to analyses in vitro. Because long range activation by enhancer elements observed in vivo is not as yet reproducible in vitro (9,40; Westin and Schaffner, unpublished results) and, since it is known that many enhancer sequence motifs function as promoter components in a position just upstream of the TATA box (G. Westin, unpublished results), we have devised the vector primarily to test the effect of such cis-acting elements in a "promoter" position, at -37 near the globin TATA box. Although the S1-nuclease mapping procedure is more time-consuming than, for example, the CAT assay, we think that our vector system has the advantage that it allows direct comparison between in vivo and in vitro results. In addition, RNA mapping of the β-globin test and reference genes enables reliable determination of the exact amount of correctly initiated transcripts. Furthermore, artifacts due to readthrough transcription originating further upstream, or to variations in transfection efficiency, are to a large extent eliminated by the OVEC system.

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NOTE ADDED IN PROOF

SP6 mapping as an alternative to S1 mapping: RNA synthesized from OVEC and OVEC-REF can also be quantitated by RNase mapping using SP6 polymerase-generated RNA as probe. For this purpose we have recently cloned the SalI/TaqI(+179) fragment from OVEC-1 into AccI/SalI digested pSP64 plasmid. The resulting clone is designated SP6βTS.

REFERENCES

1.Weaver,R.F. and Weissmann,C. (1979) Nucleic Acids Res. 7, 1175-1193.
2.Banerji,J., Rusconi,S. and Schaffner,W. (1981) Cell 27, 299-308.
3.Mellon,P., Parker,V., Gluzman,Y. and Maniatis,T. (1981) Cell 27,279-288.

4. Gorman,C., Moffat,L.F. and Howard,B.H. (1982) Mol.Cell.Biol. 2, 1044-1051.
5. Domen,J., van Leen,R.W., Lubsen,N.H. and Schoenmakers,J.G.G. (1986) Analytical Biochemistry 155, 379-384.
6. Sawadogo,M. and Roeder,,R.G. (1985) Proc.Natl.Acad.Sci.USA 82, 4394-4398.
7. Gorski,K., Carneiro,M. and Schibler,U. (1986) Cell 47, 767-776.
8. Grosschedl,R. and Birnstiel, M.L. (1982) Proc.Natl.Acad.Sci.USA 79, 297-301.
9. Sergeant,A., Bohmann,D., Zentgraf,H., Weiher,H. and Keller,,W. (1984) J.Mol.Biol. 180, 577-600.
10. Serfling,E., Jasin,M. and Schaffner,W. (1985) Trends Genet. 2, 215-219.
11. Ondek,B., Shepard,A. and Herr,W. (1987) EMBO J. 6, 1017-1025.
12. Falkner, F.G., and Zachau,H.G. (1984) Nature 310, 71-74
13. Parslow,T.G., Blair,D.L., Murphy,W.J. and Granner,D.K. (1984) Proc.Natl.Acad.Sci.USA 81, 2650-2654
14. Dierks,P., van Ooyen,A., Cochran,M.D., Dobkin,C., Reiser,J. and Weissmann,C. (1983) Cell 32, 695-706.
15. Green,M.R., Treisman,R. and Maniatis,T. (1983) Cell 35, 137-148.
16. Kuhl,D., de la Fuente,J., Chaturvedi,M., Parismoo,S., Ryals,J., Meyer,F. and Weissmann,C. (1987) Cell in press.
17. Goodbourn,S., Burstein,H. and Maniatis,T. (1986) Cell 45, 601-610.
18. Zinn,K. and Maniatis,T. (1986) Cell 45, 611-618.
19. Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) Molecular Cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, New York.
20. Vieira,J. and Messing,J. (1982) Gene 19, 259-268.
21. de Villiers,J. and Schaffner,W. (1983) In Flavell,R.A. (ed.), Techniques in the Life Sciences, B5, Nucleic Acid Biochemistry. Elsevier, Ireland.
22. Weber,H., Dierks,P., Meyer,F., van Ooyen,A., Dobkin,C., Abrescia,P., Kappeler,M., Meyhack,B., Zeltner,A., Mullen,E.E. and Weissmann,C. (1981) In ICN-UCLA Symposium on Molecular and Cellular biology, Brown,D.D. and Fox,C.F., eds. (New York:Academic Press), vol. 33, 367-385.
23. de Villiers,J., Olson,L., Tyndall,C. and Schaffner,W. (1982) Nucleic Acids Res. 10, 7965-7976.
24. Carter,A.D., Felber,B.K., Walling,M.J., Jubier,M.-F., Schmidt,C.J. and Hamer,D.H. (1984) Proc.Natl.Acad.Sci.USA 81, 7392-7396.
25. Stuart,G.W., Searle,P.F., Chen,H.Y., Brinster,R.L. and Palmiter,R.D. (1984) Proc.Natl.Acad.Sci.USA 81, 7318-7322.
26. Maxam,A.M. and Gilbert,W. (1980) Methods Enzymol. 65, 499-560.
27. Gerster,T., Matthias,P., Thali,M., Jiricny,J. and Schaffner,W. (1987) EMBO J. 6, 1323-1330.
28. Graham,F.L. and van der Eb (1973) Virology 52, 456-467.
29. Serfling,E., Lübbe,A., Dorsch-Häsler,K. and Schaffner,W. (1985) EMBO J. 4, 3851-3859.
30. Picard,D. and Schaffner,W. (1983) Proc.Natl.Acad.Sci.USA 80, 417-421.
31. Dignam,J.D., Lebovitz,R.M. and Roeder,R.G. (1983) Nucleic Acids Res. 11, 1475-1489.
32. Mantei,N., Boll,W. and Weissmann,C. (1981) Nature 281, 40-46.
33. Shaw,G. and Kamen,R. (1986) Cell 46, 659-667.
34. Schirm,S., Jiricny,J. and Schaffner,W. (1987) Genes Dev. 1, 65-74.
35. Oliphant,A.R., Nussbaum,A.L. and Struhl,K. (1986) Gene 44, 177-183.
36. Derbyshire,K.M., Salvo,J.J. and Grindley,N.D.F. (1986) Gene 46, 145-152.
37. Plon,S.E. and Wang,J.C. (1986) Cell 45, 575-580. 38.Harland,R.M., Weintraub,H. and McKnight,S.L. (1983) Nature 302, 38-43.
39. Weintraub,H., Cheng,P.F. and Conrad,K. (1986) Cell 46, 115-122.
40. Sassone-Corsi,P., Dougherty,J.P., Wasylyk,B. and Chambon,P. (1984) Proc.Natl.Acad.Sci.USA 81, 308-312.