Nucleic Acids Research, Vol. 19, No. 24 6793–6797

cDNA cloning of the HMGI-C phosphoprotein, a nuclear protein associated with neoplastic and undifferentiated phenotypes

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Received October 2, 1991; Revised and Accepted November 20, 1991 EMBL accession no. X58380

ABSTRACT

The HMGI-C protein is a nuclear phosphoprotein expressed at high levels in transformed cells. The cDNA encoding the mouse protein has been isolated and the sequence of the encoded protein shows that it is related to the HMGY and I proteins, proteins which bind in the minor groove of DNA containing stretches of A and T. The HMGI-C protein has three short highly basic domains, an acidic C-terminal domain, and potential CDC2/p34 and casein kinase II phosphorylation sites. Analysis of mRNA levels demonstrate that the HMGI-C gene is not expressed in a variety of mouse tissues but is expressed in Lewis lung carcinoma cells.

INTRODUCTION

The High Mobility Group (HMG) proteins are a class of DNA-binding proteins characterised by their high contents of basic and acid amino acids. Although originally classed together, it is now apparent that the HMG proteins can be grouped into three quite distinct families. The HMG1 and 2 type proteins, which have two highly structured DNA-binding 'HMG-boxes' and an acidic C-terminal, have been found to enhance transcription *in vitro* (1,2) and may well be members of a much larger class of regulators containing HMG-boxes (3). The second family of HMG proteins are the random-coil proteins HMG14 and 17 of unknown function (4), and the third family are the HMGI-type proteins, proteins which bind in the minor groove of AT-rich sequences. So far three members of this third family, HMGI, HMGY and HMGI-C have been identified (5–10). (These proteins have also been termed HMG D (or α), E, C (or I') in the earlier literature). Most differentiated tissues and cells have very low levels of the HMGI-type proteins compared to the other HMG proteins; for example, thyroid epithelial cells grown in culture and exhibiting a differentiated phenotype have barely detectable levels of the HMGI-type proteins. However, when such cells are transformed with viral oncogenes high levels of all three HMGI-type proteins are observed, concomitant with the cells becoming less differentiated (8,9,10). Conversely, when F9 embryocarcinoma cells are induced to differentiate with retinoic acid the expression of the HMGI-type proteins is repressed (11). Thyroid derived fibroblasts and rat-1 fibroblasts grown in culture express HMGI and Y but not HMGI-C. When transformed with viral oncogenes, HMGI-C expression is then induced (9,10). Thus viral oncogenes appear to induce HMGI-C expression, possibly as a result of inducing a less differentiated phenotype.

In the cell nucleus, the HMGI and Y proteins have been localised to AT-rich sequences associated with centromeres and telomeres where they may play a structural role (12). They have also been found associated with other regions of the genome and since HMGI/Y can activate transcription *in vitro* (13) it is possible that, when bound to such regions, they play a role in transcription processes. This is supported by the recent finding that HMGI binds to an AT-rich activator sequence upstream of the murine lymphotoxin gene (S. Fashena, R. Reeves and N. Ruddle, manuscript submitted).

The HMGI and Y proteins have been well characterised; cDNA cloning has shown that they are products of alternatively spliced mRNA species, HMGI having an additional eleven amino acids compared to HMGY (14,15). They have also been shown to be phosphorylated at multiple sites by CDC2/cyclin at metaphase and by casein kinase II in interphase (16–18). Less is known about the third protein HMGI-C except that it is a phosphoprotein with a similar size (~12000 Da) and amino acid composition to HMGI and Y (8–10). It has been found in sarcomas, carcinomas and hepatomas of human, mouse and rat origin, but its expression may be more restricted than HMGI(Y) since it is not expressed in T-cells (8–10,19,20). In this paper we describe the molecular cloning of the cDNA for mouse HMGI-C, demonstrating that HMGI-C, although similar to HMGI/Y, is the product of a different gene. The gene is expressed in Lewis lung carcinoma cells but the mRNA is undetectable in normal mouse tissues.

MATERIALS AND METHODS

Purification of HMGI-C by HPLC

HMG and histone H1 proteins were selectively extracted from mouse Lewis lung carcinoma (19) or tumours obtained from transformed rat thyroid cells (10) with 5% perchloric acid (PC-A) and fractionated by reverse phase HPLC as previously described (19) using a BioRad RP-304 column (21.5×250mm) for large scale fractionation (20 mg of crude extract).

Peptide purification and characterization

Mouse HMGI-C (50−70 μg) was digested with 25 μg of endoproteinase Glu-C (Boehringer, Mannheim) in 25 mM ammonium carbonate, pH 7.9 at 37°C for 40 h. The peptides were separated on a Waters μ Bondapak C18 column (3.9×300 mm) with a Waters C18 pre-column insert. The elution was carried out at room temperature using a gradient from 0.1% TFA in water (solvent A) to 0.1% TFA in 95% acetonitrile, 5% water (solvent B). Two peptides were obtained which were sequenced by automated Edman degradation using an Applied Biosystems gas-phase sequencer (470A). Phenylthiohydantoin derivatives were analysed by reverse-phase HPLC. Similarly two peptides were isolated and sequenced following digestion of rat HMGI-C with AspN.

RNA preparation and Northern blot analysis

Total RNA from mouse tissues and solid tumours was extracted by disrupting the tissue in lysis buffer (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.1M 2-mercaptoethanol, 0.5% N-laurylsarcosine) using a Polytron homogeniser. RNA was then prepared according to Chomczynski and Sacchi (21). Total cellular RNA (10 μg) was used for Northern analysis on 1% agarose gels containing 6.7% formaldehyde. Gels were transferred to Genescreen plus membranes (Dupont) according to the suppliers instructions. RNA was crosslinked by exposure to UV-light. Hybridisation was performed in 1M NaCl, 1% SDS in the presence of 100 μg/ml salmon sperm DNA at 68°C. The probe, labelled by random priming to a specific activity of 5×10^8 cpm/μg, was added at 5×105 cpm/ml hybridisation solution. For poly(A) selection, mAP paper (Amersham) was used.

cDNA library construction and screening

An oligodT primed cDNA library was made from Lewis lung carcinoma poly(A)+ RNA by directional cloning (22) into the lambda vector T7-T3/E-H (23). The library was screened with a degenerate oligonucleotide (41mer) designed on the basis of the mouse HMGI-C peptide sequence KXPQQVVQKKPAQE using the criteria suggested by Lathe (24). Since the second residue was not identified, it was guessed to be a lysine. The sequence of the oligonucleotide was AAGAAGCCCCA(A/G) CA(A/G) GTG GTG CA(A/G) AA(A/G) AA(A/G)CC-TGC(T/C) CA(A/G) GA. 800ng of the oligonucleotide was end-labelled with 150 μCi γ-^{32}P-ATP to a specific activity of 2×10^8 cpm/μg. 200,000 plaques of the cDNA library were screened with the probe in a hybridisation solution containing 6×SSC, 0.5% SDS, 100 μg/ml salmon sperm DNA at 55°C for 36 h. The filters were then washed twice with 2×SSC at room temperature, once with 2×SSC, 0.1% SDS at 45°C, and then once with 0.5×SSC, 0.1% SDS at 37°C, all the washes being for 5 min. The positive plaques were rescreened as above. One positive was isolated which contained the HMGI-C peptide sequence. The insert from this clone was used to screen the same library to find other positives.

DNA sequencing and sequence analysis

The cDNA was subcloned in the Bluescript KS+ plasmid (Stratagene). Nested deletions were introduced from both ends by ExoIII digestion according to the Bluescript manual. Double-stranded DNA was isolated and sequenced using the EMBL automated sequencing apparatus. The entire sequence was read on both strands and each nucleotide was read three times on average. Nucleotide and amino acid sequences analysis were carried out using the Intelligenetics sequence analysis software package.

RESULTS

Cloning of the cDNA coding for HMGI-C

HMGI-C from mouse Lewis lung carcinoma was purified to homogeneity by reverse phase chromatography. Since the N-terminal was found to be blocked the protein was digested with Glu-C endoproteinase and two peptides were sequenced (shown in Fig.2). Two peptide sequences were also obtained from rat HMGI-C. A 41mer degenerate oligonucleotide was synthesised based on the peptide sequence K[-]PQQVVQKKPAQE, taking into account codon usage in mammals and using mixed bases in the third codon position of some of the residues. This oligonucleotide was used to screen a mouse Lewis lung carcinoma cDNA library. Several positive clones were obtained but only one turned out to be a true positive in the secondary screening. The insert of this clone HMGI-C11 was 1.7 Kb long. Using the DNA of this clone as a probe two more positives were identified, HMGI-C4 and HMGI-C7, containing inserts of 1.4 and 2.3 Kb respectively (Fig.1). Clones HMGI-C4 and HMGI-C7 start 3′ of the coding region but extend further downstream than the 3′ end of the HMGI-C11 clone. Both have polyA tails at the 3′ end but without any polyadenylation signal (data not shown).

The total length of cDNA cloned (from the three clones HMGI-C11, HMGI-C4 and HMGI-C7) is about 3 Kb which is ~1 Kb shorter than the 4 Kb mRNA seen on the Northern (see below). Since HMGI-C4 and HMGI-C7 clones do not have a polyadenylation signal but have oligodA 3′ ends, it is likely that oligodT priming in the library construction occurred from internal stretches of polyA, and that the true 3′ extremity of the mRNA may be missing from the library.

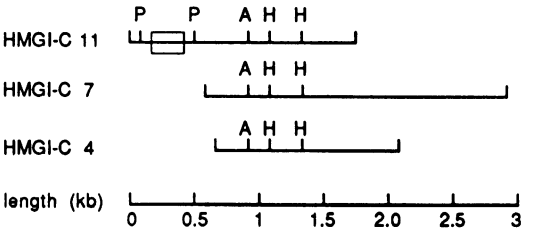

Fig. 1. Partial restriction map of three HMGI-C cDNA inserts. The boxed region indicates the amino acid coding sequence of HMGI-C. The numbers indicate the length in basepairs from the 5′ end of clone HMGI-C11. Some restriction sites are shown: P, Pst I; H, Hinc II; A, Acc I.

HMGI-C protein sequence

Figure 2 shows the amino acid sequence of HMGI-C protein deduced from the DNA sequence of the HMGI-C11 cDNA. The open reading frame encodes a protein containing the correct sequences of the two isolated mouse HMGI-C peptides. The unidentified residue at residue 10 in the second peptide is a tryptophan. Proof for the presence of tryptophan was obtained by fluorescent spectroscopic analysis of HMGI-C. These data (not shown) showed that there is one tryptophan residue in the HMGI-C protein confirming the open reading frame deduced from the cDNA. Also, HMGI-C has been shown to dimerise through disulfide linkages (10) and the single cysteine at position 41 confirms this. The open reading frame encodes a protein of 108 amino acids with an amino acid composition characteristic of the HMGI family, i.e. high levels of lysine, arginine, glutamic acid, glutamine, alanine, proline and serine and no aromatics (except for the single tryptophan found in HMGI-C). On SDS electrophoretic gels HMGI-C migrates slightly slower than HMGI which has 107 residues. Mass spectrometric data (not shown) give a value of 11,977 Da for the protein HMGI-C. The mass value obtained from the amino acid sequence of Fig.2 is 11,819 Da which makes a difference of 158 Da that could correspond to the presence of two phosphate groups bound to the serine/threonine residues. Thus the open reading frame of the cDNA clone encodes a protein of the expected size and composition of the isolated mouse HMGI-C.

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1                 10                  20                  30
M S A R G E G A G Q P S T S A Q G Q P A A P V P Q K R G R G R P

              40                  50                  60
R K Q Q Q E P T C E P S P K R P R G R P K G S K N K S P S K A A
        (mouse P1) P S P K R P R G R P K G S K N
        (rat P1) E P S P K R P R G R P K G S K N

          70                  80                  90
Q K K A E T I G E K R P R G R P R K W P Q Q V V Q K K P A Q E T
       (mouse P2) K R P R G R P R K ? P Q Q V V Q K K P A Q E
(rat P2) E P I G E K R P Q G R P R K ? P Q Q V V Q K

    100
E E T S S Q E S A E E D
```

Fig. 2. HMGI-C protein sequence deduced from HMGI-C11 cDNA clone. Underneath are shown the amino acid sequences of two peptides P1 and P2 obtained by digestion of mouse and rat HMGI-C.

As expected, high amino acid sequence homology (~50% overall) was found with HMGI and Y proteins (Fig.3). The homology is even higher in the four domains I,II,III and IV of the protein. The three basic domains I,II and III all have the eight residue motif B BXBXBXB, where B is a basic amino acid (lysine or arginine) and X is glycine or proline. This motif is that which probably interacts with DNA as suggested by Reeves and Nissen (25). The last domain IV that corresponds to the acidic C-terminal of the protein, could be involved in the interaction with other proteins (26). HMGY has been shown to be a spliced variant of HMGI, having an eleven acid deletion between domains I and II, bringing these two domains into close proximity. Comparing the sequences of Fig.3, it is apparent that protein HMGI-C also has a very similar deletion. On the other hand, the acidic domain IV is more distant from the basic domain III in the protein I-C than in the other two proteins. Most of the sequence differences between HMGI-C and HMGI/Y are located in the N-terminal region where there are 13 significant differences on the first 21 residues. It should be pointed out that from the cDNA sequences HMGI-C is encoded by a different gene to that encoding the HMGI/Y proteins.

HMGI-C is like HMGI and Y a phosphoprotein (9,10). Casein kinase II was shown to phosphorylate HMGI serines 116 and 117 (in the numbering of Fig.3) *in vitro* (18). The same sites were found phosphorylated in the HMGI protein obtained from human placenta (17). Serine 116 is conserved in HMGI-C, therefore it could be the site of phosphorylation by casein kinase

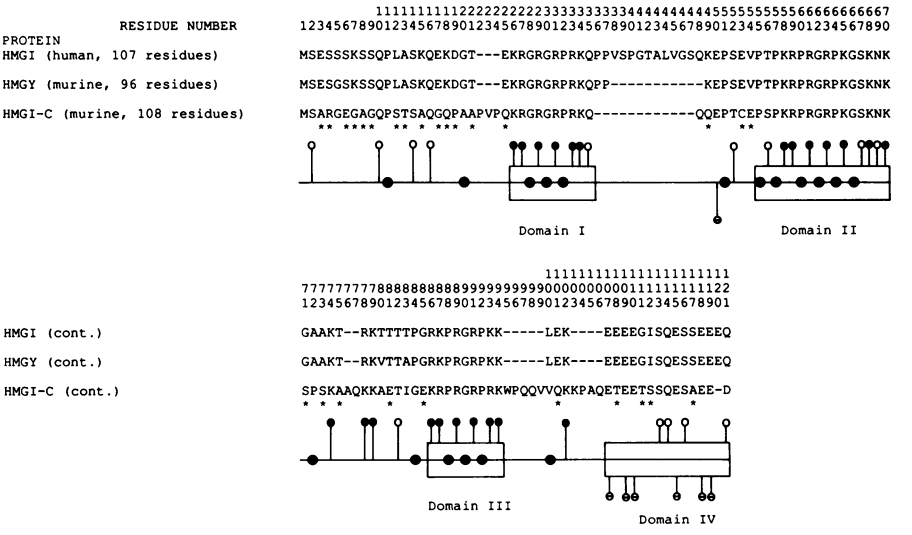

Fig. 3. Comparison between HMGI-C (this work) and HMGI and HMGY sequences. The domains I,II,III containing the conserved basic octapeptide motif and the acidic domain IV are boxed. The conserved features of the three proteins are shown.

II. It has also been shown that the CDC2 kinase phosphorylates human HMGI at the threonines 56 and 83 (Fig.3 numbering) *in vitro* (27). The threonine at position 83 of human HMGI is not conserved in murine HMGI, Y or HMGI-C. Mouse HMGI-C contains a serine residue at position 56 which could well be a site for CDC2 phosphorylation (28). From the two peptide sequences P1 and P2 obtained from rat HMGI-C (Fig.2) which span residues 54−69 and 81−102 respectively (using the numbering of Fig.3), it can be seen that the serine at residue 56 is conserved. Like mouse HMGI-C and mouse HMGI/Y the amino acid position 83 is not threonine. Also the threonine at 82 in mouse HMGI-C, which potentially could be a CDC2 phosphorylation site is not conserved in the rat HMGI-C. In conclusion, mouse and rat HMGI-C have potential sites of phosphorylation by casein kinase II and CDC2 as reported for HMGI/Y proteins.

Expression of HMGI-C mRNA in mouse tissues

The expression of HMGI-C mRNA was analysed in tissues extracted from adult Balb/c mice and compared to HMGI/Y expression. As can be seen in Figure 4, there is no detectable expression in all the tissues analysed while it is evident in the RNA sample extracted from Lewis lung carcinoma. This data on mRNA levels agrees with what is known about protein levels of HMGI-C, i.e. in normal differentiated tissues levels are very much lower than in malignant tissues. The same Northern blot was probed with the cDNA coding for HMGY which is able to recognise both HMGY and HMGI since they are very similar. The HMGI/Y mRNA expression level in Lewis lung carcinoma is considerably higher than that of HMGI-C. Furthermore a very low level of expression of HMGI/Y is detectable in some of the mouse tissues.

DISCUSSION

Cloning of the cDNA of the HMGI-C protein reveals that it is structurally homologous to the other two members of this family of proteins, HMGI and HMGY, but is expressed from a different gene. HMGI-C has three conserved basic domains and an acidic C-terminal. The domain II has a conserved motif containing a CDC2 phosphorylation site, and the C-terminal has a potential casein kinase II phosphorylation site. Although a threonine at position 83 of human HMGI (near domain III) is a second CDC2 phosphorylation site, the mouse HMGY and mouse and rat HMGI-C do not have threonines or serines at this position. There is a threonine at residue 82 in the mouse HMGI-C, but it is not known whether phosphorylation occurs at this position, and it is not present in the rat HMGI-C. Therefore phosphorylation in this region may not be important for protein function.

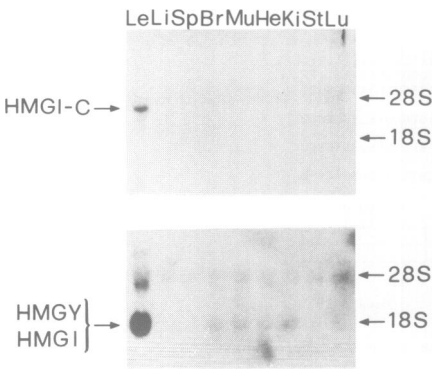

Fig. 4. Northern blot analysis of RNA extracted from Lewis lung carcinoma (Le) and various mouse tissues (Li, liver; Sp, spleen; Br, brain; Mu, muscle; He, heart; Ki, kidney; St, stomach; Lu, lung). Equal amounts (10 μg) of RNA, estimated by ethydium bromide staining, were loaded.

The HMGI-type proteins bind in the minor groove of DNA, binding preferentially to stretches of DNA containing A and T (25,29). A peptide corresponding to the basic domain II of the HMG-I type protein will on its own bind to AT DNA and a model has been proposed in which such an extended peptide with an Asx turn at one end lies in the minor groove, stabilized by hydrogen bonds and Van der Waals contacts in the minor groove and ionic interaction between the basic side chains and DNA phosphates (25). It is probable all three basic domains, I, II and III bind in the minor groove in the same way. Thus three adjacent short stretches of AT-DNA would bind the HMGI-type proteins with high affinity. It is of interest to note that the spacing between all three basic domains are conserved in HMGY and HMGI-C. The first and second boxes of HMGI are further apart due to an eleven amino acid insertion. Similar repeats are seen in the drosophila D1 protein which also has casein kinase II phosphorylation sites (30).

Phosphorylation of the threonine in the domain II weakens the binding of this peptide to DNA (31). Thus the M-phase phosphorylation of the proteins will weaken DNA binding of the HMGI-type protein (though they still remain attached to the metaphase chromosome). Similarly phosphorylation of the C-terminal by casein kinase II may affect DNA binding as has been shown for the c-myb proto-oncogene (32). It is also possible that the highly acidic C-terminal interacts with other proteins (e.g. transcription factors).

The function of the HMGI-type proteins is not clear—they have been suggested to be chromosome structural proteins (e.g. in organising satellite chromatin (6)) or transcription factors (13,33), and it is possible that they play both roles as in the case of the RAP1 protein which is bound to telomeres as well as acting as a transcription factor (34). Further evidence for a transcriptional role comes from studies on an upstream activator sequence of the murine lymphotoxin gene which binds HMGI protein (Fashena, Reeves and Ruddle, manuscript submitted).

The fact that the levels of the HMGI-type proteins are modulated by the differentiation status of the cells suggests a regulatory role for the proteins. Levels of the proteins do not correlate with cell proliferation since some cells grown in culture (e.g. normal thyroid epithelial cells) express very low levels of all three HMGI proteins compared to the levels found in transformed cells and some immortalized cell lines such as NIH-3T3 (Giancotti *et al.*, unpublished data). One possible explanation for this difference is that in normal cells the HMGI proteins are only present at some limited stage of the cell cycle whilst in transformed cells turnover may be uncoupled from the cell cycle such that the proteins are present throughout the cycle.

Although HMGI-C is similar to HMGI(Y), the expression patterns of the proteins in different cells are different in some cases, suggesting that the two genes are under different regulatory controls. For example, HMGI and Y proteins but not HMGI-C are expressed in rat-1 fibroblasts and FRT-fibroblasts, both normal cell lines (8,10). HMGI-C is only expressed when these cells are transformed with viral oncogenes. Conversely, HMGI-C but not HMGI and Y is expressed in thyroid epithelial cells

infected with murine sarcoma virus containing v-Ha-ras (10). We have also found that T-cells express HMGI and Y but not HMGI-C (20). These results would suggest that HMGI-C has a different role to that of HMGI or HMGY.

ACKNOWLEDGEMENTS

We would like to thank Ray Reeves for supplying the HMGI cDNA clone and for helpful discussions, Nancy Ruddle for communicating data on the mouse lymphotoxin gene, Grace Poon for the mass spectrometric analysis of HMGI proteins, and M. Callahan for manuscript preparation. This research was funded by grants from the *Associazione Italiana per la Ricerca sul Cancro* (AIRC), Milano,Italy; *Consiglio Nazionale delle Ricerche* (CNR), Roma, Italy; *Ministero dell'Università e della Ricerca Scientifica e Tecnologica*, Roma, Italy; The Cancer Research Campaign, UK; and the European Economic Community Stimulation Action Programme.

REFERENCES

1. Watt, F. and Molloy, P.L. (1988) *Nucleic Acids Res.*, **16**, 1471–1486.
2. Singh, J. and Dixon, G.H. (1990) *Biochemistry*, **29**, 6295–6302.
3. Jantzen, H., Admon, A., Bell, S.P. and Tjian, R. (1990) *Nature* **344**, 830–836.
4. Goodwin, G. and Bustin, M. (1988) in *Architecture of eukaryotic genes* (Kahl, G., ed.), pp.187–205, VCH, Weinheim.
5. Lund, T., Holtlund, J., Fredriksen, M. and Laland, S.G. (1983) *FEBS Lett.*, **152**, 163–167.
6. Strauss, F. and Varshavsky, A. (1984) *Cell*, **37**, 889–901.
7. Lund, T., Holtlund, J. and Laland, S.G. (1985) *FEBS Lett.*, **180**, 275–279.
8. Goodwin, G.H., Cockerill, P.N., Kellam, S. and Wright, C.A. (1985) *Eur. J. Biochem.*, **149**, 47–51.
9. Giancotti, V., Berlingieri, M.T., Di Fiore, P.P., Fusco, A., Vecchio, G. and Crane-Robinson, C. (1985) *Cancer Res.*, **45**, 6051–6057.
10. Giancotti, V., Pani, B., D'Andrea, P., Berlingieri, M.T., Di Fiore, P.P., Fusco, A., Vecchio, G., Philp, R., Crane-Robinson, C., Nicolas, R.H., Wright, C.A. and Goodwin, G.H. (1987) *EMBO J.*, **6**, 1981–1987.
11. Vartiainen, E., Palvimo, J., Mahonen, A., Linnala-Kankkunen, A. and Maenpaa, P.H. (1988) *FEBS Lett.*, **228**, 45–48.
12. Disney, J., Johnson, K.R., Magnuson, N.S., Sylvester, S.R. and Reeves, R. (1989) *J. Cell. Biol.*, **109**, 1975–1982.
13. Yang-Yen, H.F. and Rothblum, L.I. (1988) *Mol. Cell. Biol.*, **8**, 3406–3414.
14. Johnson, K.R., Lehn, D.A., Elton, T.S., Barr, P.J. and Reeves, R. (1988) *J. Biol. Chem.*, **263**, 18338–18342.
15. Johnson, K.R., Lehn, D.A. and Reeves, R. (1989) *Mol. Cell. Biol.*, **9**, 2114–2123.
16. Lund, T. and Laland, S.G. (1990) *Biochem. Biophys. Res. Commun.*, **171**, 342–347.
17. Lund, T., Dahl, K.H., Mork, E., Holtlund, J. and Laland, S.G. (1987) *Biochem. Biophys. Res. Commun.*, **146**, 342–347.
18. Palvimo, J. and Linnala-Kankkunen, A. (1989) *FEBS Letts.*, **257**, 101–104.
19. Giancotti, V., Buratti, E., Perissin, L., Zorzet, S., Balmain, A., Portella, G., Fusco, A. and Goodwin, G.H. (1989) *Exp. Cell. Res.*, **184**, 538–545.
20. Giancotti, V., Bandiera, A., Buratti, E., Fusco, A., Marzari, R., Coles, B. and Goodwin, G.H. (1991) *Eur. J. Biochem.*, **198**, 211–216.
21. Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.*, **162**, 156–159.
22. Dorssers, L. and Postmes, A. (1987) *Nucleic Acids Res.*, **15**, 3629.
23. Grimaldi, G., Manfioletti, G. and Schneider, C. (1987) *Nucleic Acids Res.*, **15**, 9608.
24. Lathe, A. (1985) *J. Mol. Biol.*, **183**, 1–12.
25. Reeves, R. and Nissen, M.S. (1990) *J. Biol. Chem.*, **265**, 8573–8582.
26. Ptashne, M. (1988) *Nature*, **335**, 683–689.
27. Lund, T. and Laland, S.G. (1990) *Biochem. Biophys. Res. Comm.*, **171**, 342–347.
28. Draetta, G. (1990) *TIBS*, **15**, 378–383.
29. Solomon, M.J., Strauss, F. and Varshavsky, A. (1986) *Proc. Natl. Acad. Sci. (USA)*, **83**, 1276–1280.
30. Ashley, C.T., Pendleton, C.G., Wesley Jennings, W., Saxena, A. and Glover, C.V.C. (1989) *J. Biol. Chem.*, **264**, 8394–8401.
31. Reeves, R., Langan, T.A. and Nissen, M.S. (1991) *Proc. Natl. Acad. Sci. (USA)*, **88**, 1671–1675.
32. Luscher, B., Christenson, E., Litchfield, D.W., Krebs, E.G. and Eisenman, R.N. (1990) *Nature*, **344**, 517–522.
33. Reeves, R., Elton, T.S., Nissen, M.S., Lehn, D.A. and Johnson, K.R. (1987) *Proc. Natl. Acad. Sci. (USA)*, **84**, 6531–6535.
34. Devlin, C., Tice-Baldwin, K., Shore, K. and Arndt, K. (1991) *Mol. Cell. Biol.*, **11**, 3642–3651.