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Tissue specific expression of avian vitellogenin gene is correlated with DNA hypomethylation and *in vivo* specific protein–DNA interactions

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The avian vitellogenin gene is expressed only in the liver of egg-laying hens. It can, however, be activated in immature chicks or roosters by oestradiol. Parallel to the onset of transcription, there is a demethylation of specific mCpGs in the promoter region and in the oestrogen response element (ERE). The methylation pattern in the promoter region is hormone and expression specific, whereas in the ERE it is only hormone and not organ specific. The demethylation occurring in the promoter region is correlated with the appearance of DNase I hypersensitivity sites and changes in the specific protein–DNA interactions. *In vivo* genomic footprinting of the ERE with varying concentrations of dimethylsulphate revealed, upon gene activation, only minor changes in the protein–DNA interaction. We present evidence that there is another protein that binds with high affinity to the ERE, besides the oestrogen receptor.

1. Introduction

Vitellogenin is one of the major yolk proteins synthesized in the liver of egg-laying vertebrates (for review, see Tata *et al.* (1987); Wahli (1988) and references therein). Vitellogenin in chickens is encoded by three different genes (Wang *et al.* 1983), whereas in *Xenopus* the yolk protein is coded by three small gene families (Wahli 1988). In vertebrates, vitellogenin genes are under the control of oestradiol (Tata *et al.* 1987; Wahli 1988). The silent genes in immature animals and adult roosters can be stimulated by a single injection of oestradiol (Deeley *et al.* 1977; Jost *et al.* 1978). In chick embryos the vitellogenin II gene is activated by injections

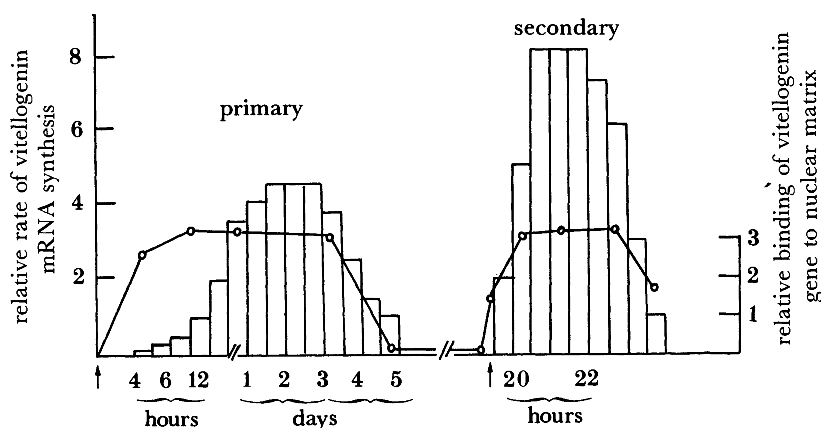

Figure 1. The primary and secondary stimulation of avian vitellogenin gene by oestradiol. Immature chicks were treated with a single injection of oestradiol (40 mg kg^{-1} body mass) (see vertical arrows). Bar diagram represents the relative level of vitellogenin mRNA as measured by molecular hybridization with a labelled cDNA probe. The solid line represents the binding of the 5′ end flanking region of the gene to the nuclear matrix.

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of oestradiol into the yolk sack two days after the first appearance of hepatic oestradiol receptor (Lazier 1978). Upon injection of oestradiol to immature chicks (see figure 1) there is a lag of about 12 h before the first appearance of vitellogenin mRNA (primary stimulation). About ten days after the primary stimulation, vitellogenin mRNA levels return to background levels. If a second dose of oestradiol is given at this time (secondary stimulation), the vitellogenin mRNA is immediately synthesized at a maximal rate (Jost *et al.* 1978; Ryffel *et al.* 1977). This memory effect has been correlated with the appearance and persistance of DNase I hypersensitivity sites in the promoter region (Burch & Weintraub 1983; Burch 1984; Burch & Evans 1986; see also figure 2) and also to the demethylation of specific mCpGs within the DNase I hypersensitive site of the promoter and oestrogen-response element (Saluz *et al.* 1986; Saluz *et al.* 1988).

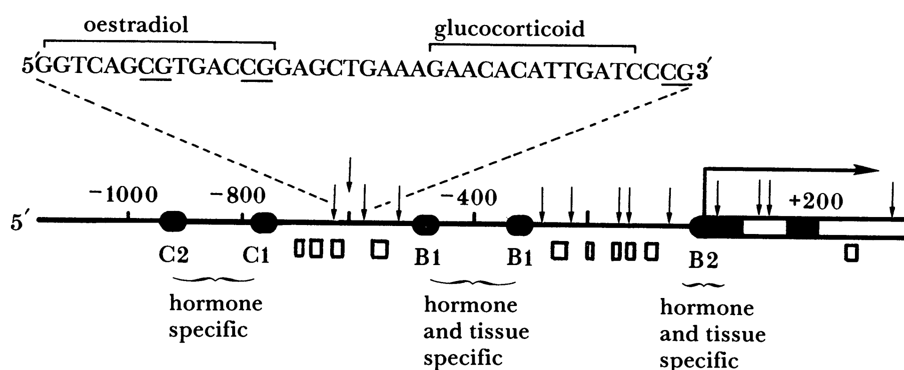

FIGURE 2. Organization of the upstream region of avian vitellogenin II gene. B1, B2, C1 and C2 are the DNase I hypersensitive sites which appear upon oestradiol treatment. The open boxes below the horizontal line are the A+T rich regions (80% A+T). The arrows pointing downward represent the CpGs.

The hormonal stimulation of vitellogenin mRNA synthesis requires neither DNA nor protein synthesis (Wilks *et al.* 1984; Hayward *et al.* 1982), however, it is preceded by the association of the 5′ and 3′ end flanking regions of the gene to the nuclear matrix (Jost & Seldran 1984, see also figure 1). This association to the nuclear matrix is tissue specific and reversible. In this review, we show that oestradiol changes the methylation pattern of specific CpGs in the upstream region of the gene and that in parallel with the demethylation events there are also specific changes in the protein–DNA interactions *in vivo* and *in vitro*.

2. Demethylation of an mCpG in the promoter region is organ and expression specific

Several lines of evidence suggest that DNA methylation plays a role in gene regulation (Razin *et al.* 1984; Adams & Burdon 1985). Studies of numerous tissue-specific genes using methylation-specific restriction enzymes have shown a clear correlation between the methylation levels of active and inactive genes. In the case of vitellogenin gene the state of methylation of the CpG situated in the promoter region could not be studied by methylation-sensitive restriction enzymes, therefore, we used genomic sequencing, first described by Church & Gilbert (1984), and improved by Saluz & Jost (1986, 1987). Figure 3 shows a short outline of the basic principles of genomic sequencing.

Total genomic DNA is cut with a suitable restriction enzyme and upon partial reaction with hydrazine and cleavage with piperidine, the reaction product is separated on a sequencing gel.

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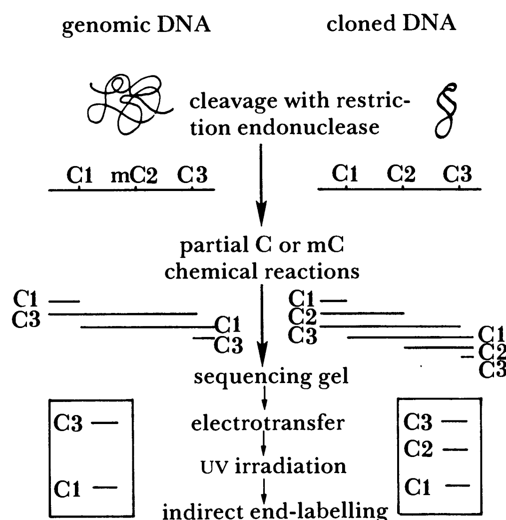

FIGURE 3. Genomic sequencing scheme. The indirect end labelling is done by hybridizing the filters with a labelled single-stranded DNA or RNA probe complementary to one end of the restriction fragments.

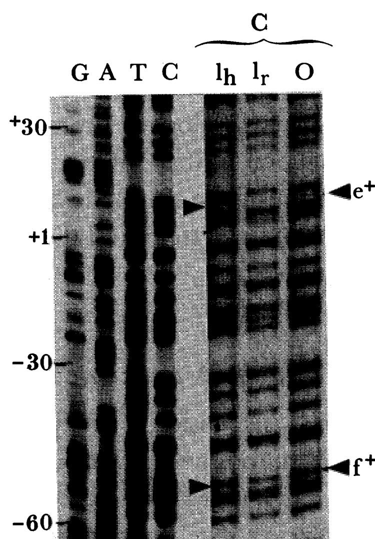

FIGURE 4. The demethylation of mCpG in the promoter region of vitellogenin gene is expression specific. Genomic sequencing was carried out as outlined by Saluz & Jost (1987). C-specific reactions were carried out with total DNA from hen liver (l_h), rooster liver (l_r) and hen oviduct (o). Arrows e^+ and f^+ indicate the position of CpGs.

After electrotransfer of the DNA fragments onto a nylon membrane, the DNA is covalently attached to the membrane by ultraviolet irradiation. The sequence is then visualized by indirect end-labelling (hybridization with a labelled single-stranded DNA fragment) and subsequent autoradiography of the filter. As methylated cytosine does not react with hydrazine, it is not cleaved by piperidine and thus generates a gap in the C-sequencing ladder. Such a gap could also represent a base transition caused by the deamination of 5-methylcytosine to thymidine. Therefore it is necessary to carry out a T-specific reaction as a control (Saluz *et al.* 1986). Figure 4 shows the results obtained by genomic sequencing for the promoter region. It can be seen that the CpG at nucleotide position −52 is not methylated regardless of whether the gene is expressed or not. For the CpG at nucleotide position +10,

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avian vitellogenin II		5′ TCACCTTCGCTATGAGGGGGA 3′
Xenopus laevis vitellogenin	A1	5′ TCGCCATCACCATGAGGGGAA 3′
	A2	5′ TCACCATCACCATGAAGGGAA 3′
	B1	5′ TCGCCATCACCATGAGGGGAA 3′
	B2	5′ TCACCATCACGATGAGGGGAA 3′

FIGURE 5. Sequence homology between the promoter region of chicken and *Xenopus laevis* vitellogenin genes. The CpGs are underlined.

however, there is a clear correlation between the demethylation of the mCpG and the activation state of the gene. In the oviduct this CpG remains methylated (Saluz *et al.* 1988). Kinetic studies have shown that both strands were demethylated at the same time, with the onset of vitellogenin mRNA synthesis. It is interesting that this site is situated in the middle of the DNase I hypersensitive site B2 that was described by Burch & Weintraub (1983). A computer search has shown that in the promoter region there is high homology between the different vitellogenin genes from chicken and *Xenopus*. Figure 5 shows that although the position of this CpG varies from gene to gene, it is always located within the region in which specific proteins bind. To see whether the methylation of this particular CpG plays a role in transcription, gene expression in a heterologous transcription system has been tested. Preliminary results show that methylation of the CpG at position +10 completely suppressed *in vitro* transcription (HeLa cell lysate) of the synthetic gene. This experiment is now being repeated using a homologous transcription system and dinucleotide CpG at various positions within the protein binding site.

3. *In vivo* and *in vitro* protein–DNA interaction in the promoter region

It has been shown that when a cell suspension is treated with dimethylsulphate there is a selective reaction of the unprotected guanosine residues in the native chromatin. Such *in vivo* footprinting allows the study *in situ*, of changes in the protection of DNA by proteins (Nick & Gilbert 1985; Ephrussi *et al.* 1985; Becker *et al.* 1986; Gimble & Max 1987; Pauli *et al.* 1987; Becker *et al.* 1986; Saluz *et al.* 1988). By treating a cell suspension with different concentrations of dimethylsulphate it was possible to determine the end-point titration of defined guanosine residues in the promoter region of inactive and active vitellogenin gene (Saluz *et al.* 1988). The resulting footprints show that in rooster liver at least 10 base pairs, including the methylated CpG, were protected (see figure 6 and its interpretation diagram in figure 7). In contrast, in hen liver cells, where the gene is active, the same CpG demethylation site lies within a protected region of about 20 base pairs. This means that upon activation of the gene there is a demethylation of one CpG, together with the appearance of a DNase I hypersensitive site and a change in the pattern of the *in vivo* protein–DNA interaction. The results of such *in vivo* experiments were also confirmed *in vitro*. The interaction of nuclear proteins with end-labelled double-stranded synthetic oligonucleotide (nucleotide positions −12 to +61) was tested *in vitro* using DNase I protection experiments. The results given in figure 8 show the same position of DNA protection as achieved *in vivo* (Saluz *et al.* 1988). An oligonucleotide corresponding to the protected area was then used for gel shift assays with the nuclear extracts from egg-laying hens

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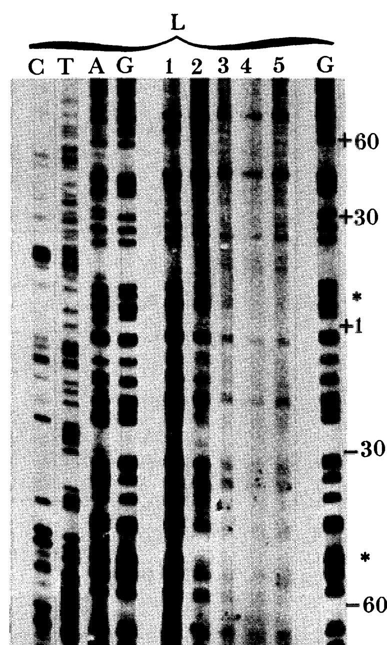

FIGURE 6. Example of an *in vivo* footprinting of rooster hepatocytes with dimethylsulphate. We show only the lower strand (L) of the promoter region of vitellogenin gene. Decreasing concentrations of dimethylsulphate (lanes 1–5: 0.5 %, 0.05 %, 0.005 %, 0.0005 %, 0.00005 % (by volume), respectively) were used to reveal the details of the footprint. The full star represents the position of the methylated CpG and the open star the non-methylated CpG.

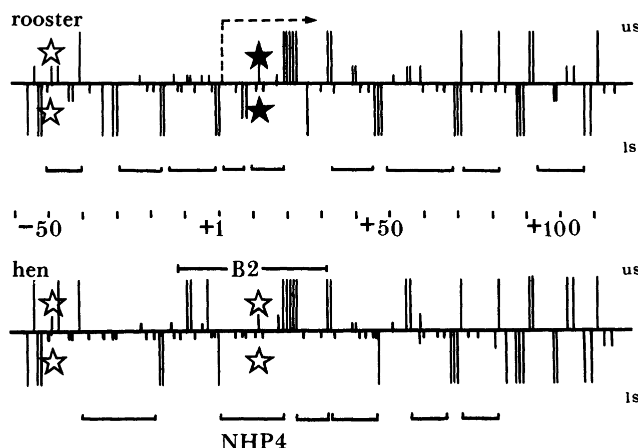

FIGURE 7. Graphic representation of the relative extent of the reaction of dimethylsulphate with guanosine residues. Vertical bars above each horizontal line represent the upper DNA strand (us) and lines below represent the lower DNA strand (ls). Length of the vertical bars indicate the relative strength of the autoradiogram signals. B2 is the tissue-specific and expression-specific DNase I hypersensitive site (Saluz *et al.* 1988).

and roosters. Figure 8 shows that the double-stranded sequence 5′ TCACCTTCGCTATG 3′ binds proteins from both hen and rooster livers, oviduct, HeLa and MCF-7 nuclear extracts. However, the binding activity is more abundant in hen than in rooster liver. If the cytosine at position +10 is methylated (as it is in rooster liver where the vitellogenin gene is not expressed), the double-stranded oligonucleotide binds only a protein (fraction 0.5 M potassium chloride (KCl) from heparin Sepharose) from rooster and not from hen liver. When tested for single-stranded DNA binding activity, the nuclear extract from egg-laying hens had higher levels of

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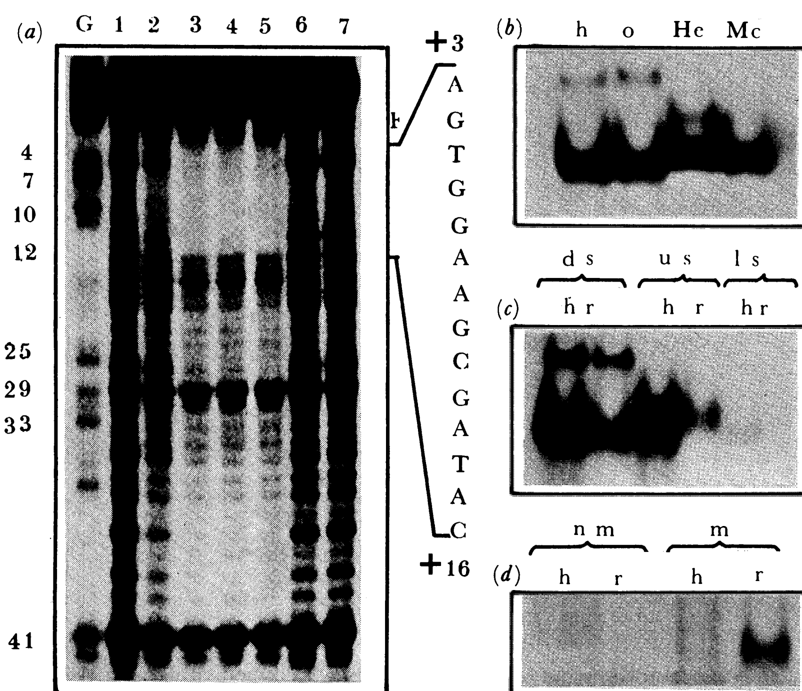

FIGURE 8. DNase protection in the region of the transcriptional start of the vitellogenin gene and gel shift assays. (*a*) DNase I protection experiment was performed on the lower strand of an oligonucleotide duplex (nucleotide position −12 to +61) using a 0.5 M heparin Sepharose fraction of HeLa cell lysate. Gel-shift assay (only the protein–DNA complexes are shown) shows (*b*) complexes between labelled oligonucleotide (positions +3 to +16) with nuclear proteins from hen liver (h), oviduct (o), HeLa cell (He) and MCF-7 cells (Mc). (*c*) Complex formed between the same labelled oligonucleotide as above with hen liver (h) or rooster liver (r) nuclear extracts. Abbreviations: ds, double-stranded DNA; us and ls upper and lower single-stranded DNA, respectively. (*d*) Complex between the labelled oligonucleotide duplex (positions +3 to +29) with hen (h) or rooster liver (r) nuclear fraction (eluted from heparin Sepharose with 0.5 M KCl). Abbreviations: nm and m, non-methylated and methylated oligonucleotides, respectively (Saluz *et al.* 1988).

binding activity than did that from rooster. In addition, only the message-like DNA strand bound the protein. Recent results show that the protein with single-stranded binding activity can be separated from that with double-stranded binding activity using chromatography on heparin Sepharose. Further experiments have shown that the single strand DNA- and also RNA-binding activity is more abundant in the cytoplasm than in the nucleus and that the protein is oestrogen-dependent (Feavers *et al.*, unpublished data). Taken together, these results show that the same region of the promoter binds at least three different proteins: one is male specific and binds only to the methylated DNA; the second one is ubiquitous and binds only non-methylated double-stranded DNA; the third one has a high affinity for the single-stranded message-like DNA and RNA.

4. Demethylation in the oestrogen-response element is oestrogen dependent but not organ specific

Previous experiments in which restriction endonucleases *Hpa*II/*Msp*I were used have indicated that treatment of immature chicks with oestradiol results in demethylation of a CpG situated in the ERE (Wilks *et al.* 1982). As other CpGs in the upstream region of the gene are

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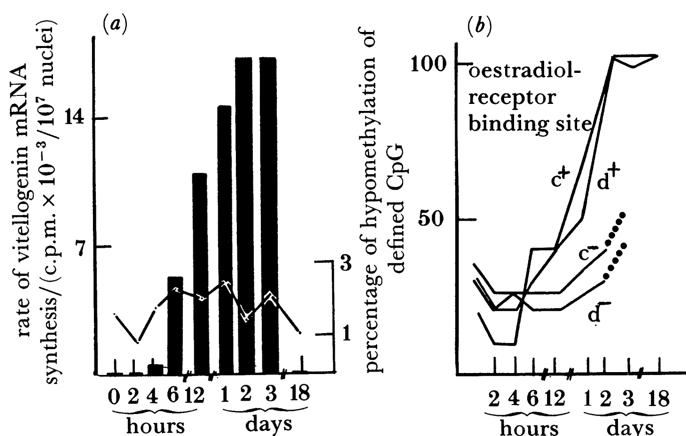

FIGURE 9. Summary of the kinetics of vitellogenin mRNA synthesis and the strand-specific demethylation of the oestrogen response element of avian vitellogenin gene. (*a*) Shows the relative rate of vitellogenin mRNA synthesis after primary stimulation with a single dose of oestrogen. The kinetics (*b*) of the relative amount of demethylation (percentage) of the CpGs c and d situated in the oestrogen-response element were determined by genomic sequencing. The (+) and (−) signs represent the upper and lower DNA strands, respectively (Saluz *et al.* 1986); (—·—) shows the relative rate of DNA synthesis (c.p.m. $\times 10^{-3}$ per 10^7 nuclei).

not recognized by restriction enzymes, we used genomic sequencing to study their methylation states (Saluz *et al.* 1986). Figure 9 shows a summary of the kinetics of demethylation of the two CpGs situated in the ERE. With oestradiol treatment the demethylation of the upper DNA strand correlates well with the induction of vitellogenin mRNA synthesis, whereas the demethylation of the lower DNA strand lagged by about 24 h (Saluz *et al.* 1986)

5. *In vivo* and *in vitro* protein–DNA interaction at the oestrogen-response element

The *in vivo* interactions of proteins with the ERE was tested by means of dimethylsulphate footprinting. For the promoter region we varied the concentration of dimethylsulphate from 0.5–0.0005% (by volume) final concentrations. A summary of the data obtained by such *in vivo* footprinting of the ERE in hepatocytes of egg-laying hens versus that of roosters is given in figure 10. In both cases, the ERE was protected by proteins, however, some minor differences in the reaction of the guanosine residues was observed in the spacer in the middle of the palindromic sequence.

When adult roosters were injected with oestradiol the pattern of protection became identical to that observed in hens. In experiments where we used specific monoclonal antibodies against the oestrogen receptor, we could also demonstrate that another protein, which differs from the oestrogen receptor, binds to the ERE (McEwan *et al.*, submitted). Gel shift assays and reconstitution experiments (Feavers *et al.* 1987) indicated that a protein other than the oestradiol-receptor bound tightly to the ERE. Pre-saturation of the ERE with this protein, NHP1, increases the binding of the labelled oestradiol receptor to the ERE (Feavers *et al.* 1987). Recently, this protein has been purified to homogeneity from HeLa cells and characterized (Hughes *et al.*, submitted). It consists of two polypetides of molecular mass 75 and 85 kDa and binds to the ERE with a K_d of 10^{-11} M. Microsequence analysis of peptides

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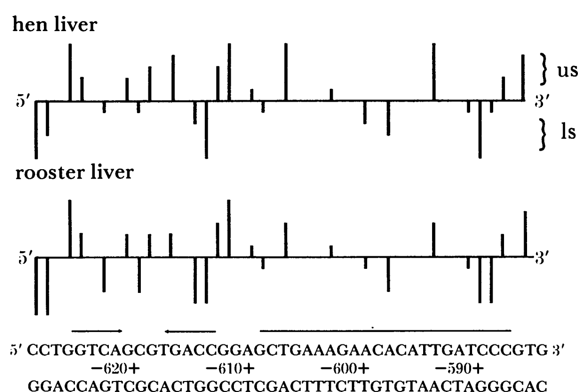

FIGURE 10. Interpretation diagram of the *in vivo* footprinting of the oestrogen-response element of vitellogenin gene in egg-laying hens (active gene) and rooster (inactive gene) hepatocytes. Abbreviations: us, upper DNA strand; ls, lower DNA strand. The length of the vertical lines gives an estimate of the degree of reactivity of each guanosine with dimethylsulphate, the smaller the bar the more protected is the base.

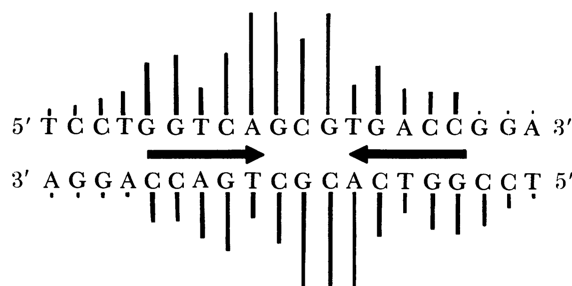

FIGURE 11. Summary of contact points of NHP1 with the oestrogen-response element. The length of the vertical lines indicate the importance of the corresponding base for the binding of NHP1. The horizontal arrows emphasize the dyad symmetry structure of the oestrogen-response element.

generated by (CNBr) fragmentation and tryptic digestion indicated that this protein is not related to the oestrogen receptor. In addition, NHP1 does not bind oestradiol and does not cross-react with antibodies directed against the oestrogen receptor. The contact points of NHP1 with the ERE were tested by partial depurination and depyrimidination of the synthetic ERE. After protein–DNA interaction, the reaction product was separated on a native agarose gel and the protein DNA complex and free DNA were extracted, purified, subjected to piperidine reaction and analysed on a sequencing gel. Figure 11 shows an interpretative diagram of the original results (Hughes *et al.*, submitted).

As shown in figure 11, the strongest contact points between the NHP1 and the ERE are located in the middle of the ERE. The protein appears to contact the DNA by binding preferentially to purine bases. Similar results were obtained by DNA competition assays, where the suppression of the three bases in the centre of the palindrome decreased the binding of the protein by 90% (Feavers *et al.* 1987).

6. DISCUSSION

Keshet *et al.* (1986) have shown that when *in vitro* methylated genes were transfected into mouse L cells, no DNase I hypersensitive sites were observed, although they were present when the corresponding unmethylated genes were transfected into L cells. Similarly,

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nuclease-hypersensitive sites in hypomethylated regions of active X-linked housekeeping genes have been described by several groups (Hansen *et al.* (1988) and references therein). In agreement, we find that in the promoter of the avian vitellogenin II gene, there is a positive correlation between its hormone-specific activation, the demethylation of one CpG and the appearance of the DNase I hypersensitive site B2. Such a correlation between demethylation and the presence of a DNase I hypersensitivity site does not, however, exist in the oestrogen-response element present in the upstream region of avian vitellogenin gene. Nevertheless, the inhibition of total DNA synthesis by either hydroxyurea or cytosine arabinoside did not prevent vitellogenin mRNA synthesis and the demethylation of mCpG (Wilks *et al.* 1984). This suggests that mCpG demethylation may not be explained by the inhibition of a replication-dependent repair methylase. Limited DNA replication, such as gene amplification that might account for passive demethylation, had been excluded by gene titration experiments carried out before and after oestradiol treatment (Jost *et al.* 1977). Two plausible mechanisms of demethylation remain: active demethylation by excision repair, by activity of a demethylase, or both (Gjerset & Martin 1982). An enzymic removal of a methyl group from 5-methyl-cytosine in DNA is mechanistically improbable because the transfer of the methyl group from S-adenosine-L-methionine to a carbon atom is irreversible at a neutral pH. However, Razin *et al.* (1986) have recently shown that in differentiating Friend erythroleukaemia cells, a replacement of 5-methylcytosine with cytosine probably occurs via an enzymic pathway. In our case, all attempts to reconstitute such a repair mechanism *in vitro* have failed. So far as the demethylation of the ERE is concerned, the question is whether the oestradiol receptor plays a role in its demethylation. Kumar *et al.* (1988) have shown that the activated oestradiol receptor binds *in vitro* with high affinity as a dimer to the ERE. The distal GG situated at the end of the palindrome plays a major role for the tight binding of the receptor to DNA. On the other hand, our results indicate that another protein, NHP1, binds with high affinity (K_d 10^{-11} M) to the ERE and the strong contact points between this protein and DNA are different than those of the oestradiol receptor. NHP1 has the highest affinity for the nucleotides situated in the spacer region of the palindrome (which contains the CpG) (Hughes *et al.*, submitted). At this point, the exact relation between NHP1 and the oestrogen receptor is still unknown. NHP1 could tag the chromatin, showing the oestradiol receptor where to bind or it could be directly involved in CpG demethylation. The observation that pre-saturation of DNA with NHP1 facilitates the binding of the labelled oestrogen–receptor complex to DNA (Feavers *et al.* 1987) supports the first hypothesis. We have, as yet, no evidence that upon binding of the oestrogen receptor, NHP1 remains bound to the DNA. As already shown by Burch & Weintraub (1983), in the liver of control and oestradiol-treated immature chicks, there are no DNase I hypersensitive sites in the ERE, indicating that this region is always protected by proteins. Similarly, *in vivo* footprinting of the ERE with dimethylsulphate has recently shown that the distal ERE present in the upstream region of vitellogenin gene is always occupied by protein. (McEwan *et al.*, submitted). It is most likely that the mechanism of active demethylation during gene activation is universal and is neither tissue nor hormone specific. In addition, it probably involves several enzymes. As NHP1 is ubiquitous and can be found in all tissues tested, it might play a role in active demethylation of DNA. Experiments to test this hypothesis are in progress.

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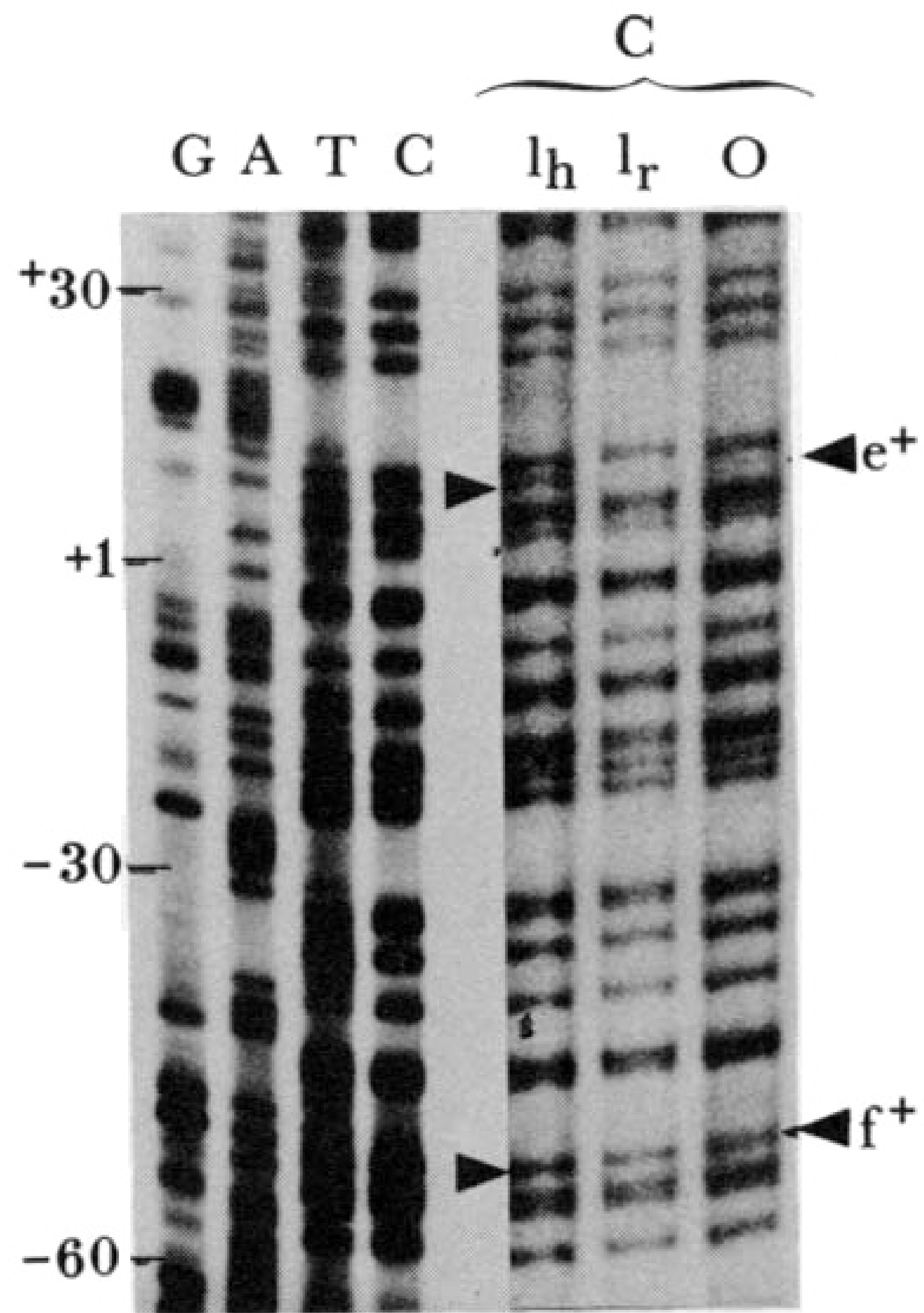

FIGURE 4. The demethylation of mCpG in the promoter region of vitellogenin gene is expression specific. Genomic sequencing was carried out as outlined by Saluz & Jost (1987). C-specific reactions were carried out with total DNA from hen liver (l_h), rooster liver (l_r) and hen oviduct (o). Arrows e^+ and f^+ indicate the position of CpGs.

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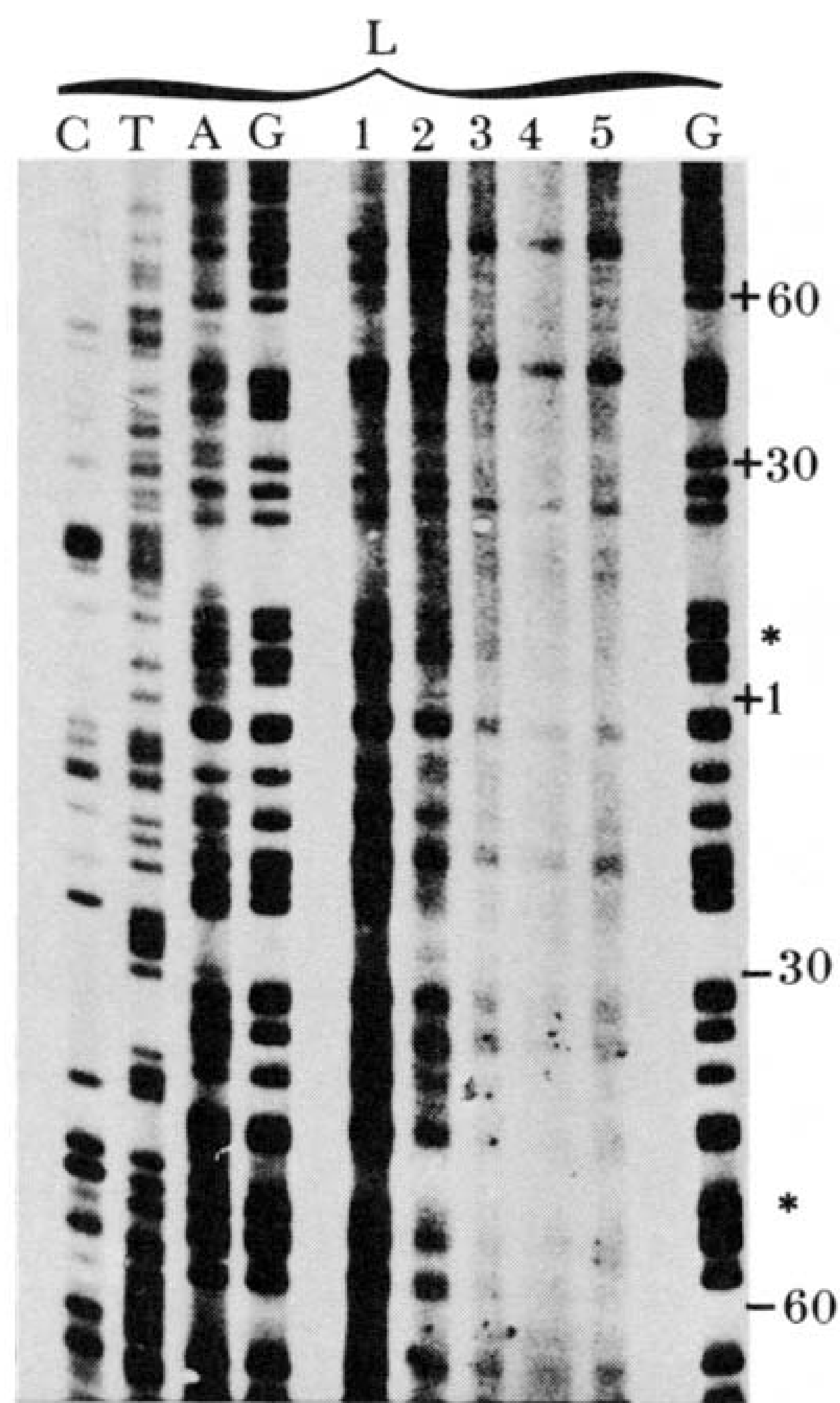

GURE 6. Example of an *in vivo* footprinting of rooster hepatocytes with dimethylsulphate. We show only the lower strand (L) of the promoter region of vitellogenin gene. Decreasing concentrations of dimethylsulphate (lanes 1–5: 0.5 %, 0.05 %, 0.005 %, 0.0005 %, 0.00005 % (by volume), respectively) were used to reveal the details of the footprint. The full star represents the position of the methylated CpG and the open star the non-methylated CpG.

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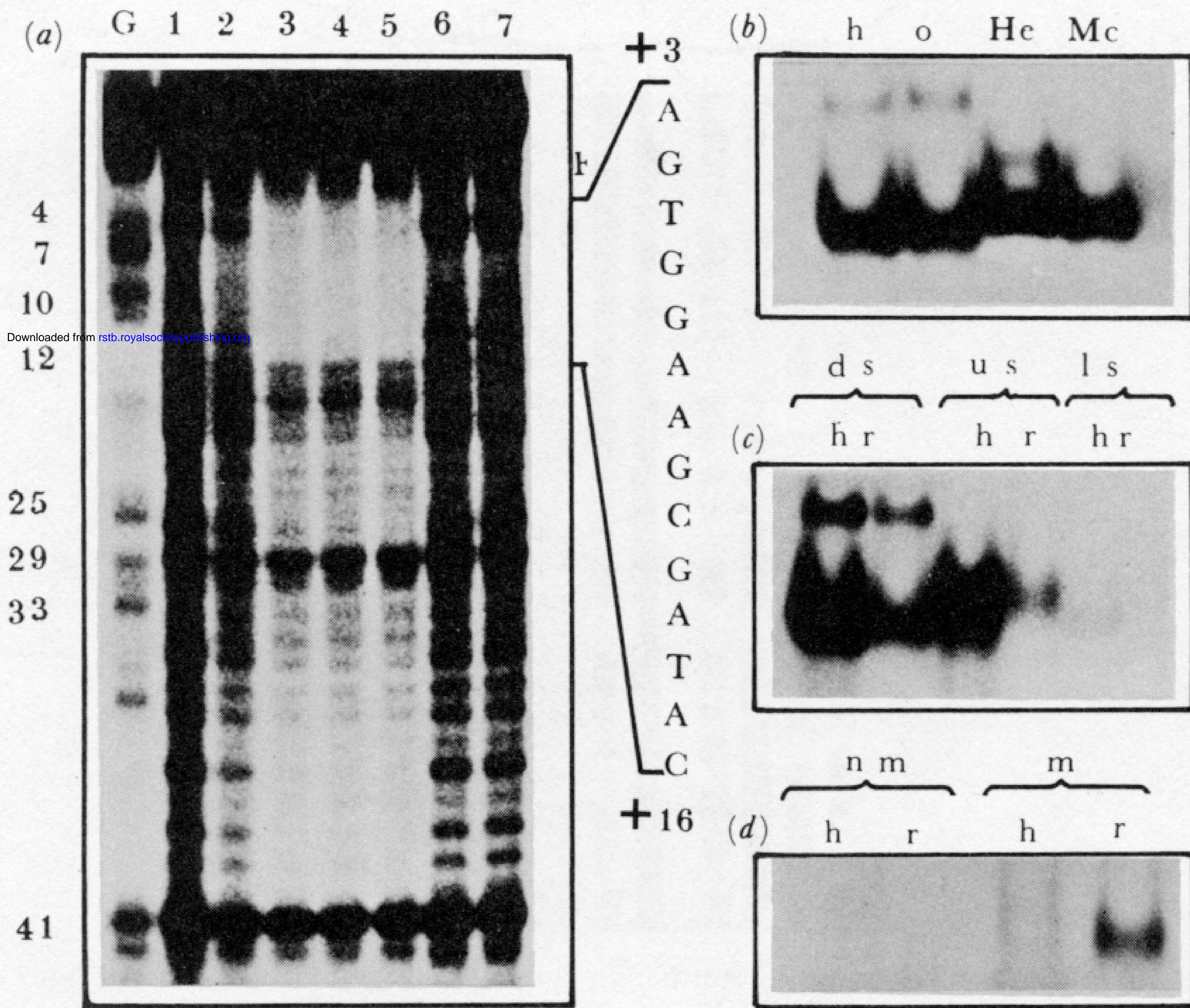

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IGURE 8. DNase protection in the region of the transcriptional start of the vitellogenin gene and gel shift assays. (*a*) DNase I protection experiment was performed on the lower strand of an oligonucleotide duplex (nucleotide position −12 to +61) using a 0.5 M heparin Sepharose fraction of HeLa cell lysate. Gel-shift assay (only the protein–DNA complexes are shown) shows (*b*) complexes between labelled oligonucleotide (positions +3 to +16) with nuclear proteins from hen liver (h), oviduct (o), HeLa cell (He) and MCF-7 cells (Mc). (*c*) Complex formed between the same labelled oligonucleotide as above with hen liver (h) or rooster liver (r) nuclear extracts. Abbreviations: ds, double-stranded DNA; us and ls upper and lower single-stranded DNA, respectively. (*d*) Complex between the labelled oligonucleotide duplex (positions +3 to +29) with hen (h) or rooster liver (r) nuclear fraction (eluted from heparin Sepharose with 0.5 M KCl). Abbreviations: nm and m, non-methylated and methylated oligonucleotides, respectively (Saluz *et al.* 1988).