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# Cell-specificity of the chicken ovalbumin and conalbumin promoters

**Andrée Dierich, Marie-Pierre Gaub, Jean-Paul LePennec, Daniel Astinotti and Pierre Chambon**

Laboratoire de Génétique Moléculaire des Eucaryotes du CNRS, Unité 184 de Biologie Moléculaire et de Génie Génétique de l'INSERM, Institut de Chimie Biologique, Faculté de Médecine, 11, Rue Humann, 67085 Strasbourg Cédex, France

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**A series of recombinant plasmids containing increasing lengths of the 5′-flanking promoter sequences of the chicken conalbumin and ovalbumin genes fused to the sequences coding for the SV40 T-antigen have been constructed. These recombinants were introduced into a variety of established cell lines and primary cultured cells by nuclear microinjection. Promoter activity was estimated by monitoring T-antigen synthesis by indirect immunofluorescence. We show that the microinjected ovalbumin and conalbumin promoter regions do not function in chicken fibroblasts, kidney cells and in a variety of non-chicken cells, irrespective of the presence of steroid hormone receptors. In contrast, these promoter regions are active in primary cultured chicken embryonic hepatocytes and oviduct tubular gland cells, suggesting the presence of cell-specific transcription factors in these cells. Unexpectedly, promoter sequences close to the TATA boxes of both the ovalbumin and conalbumin genes are sufficient to confer cell-specific expression. Most of the controls exerted on the ovalbumin and conalbumin promoters in the whole animal appear to be reproduced *in vitro* by nuclear microinjection of the chimeric genes into the primary cultured cells. However, the microinjected ovalbumin promoter is active in embryonic hepatocytes and thus escapes the regulation imposed on the corresponding inactive endogenous gene.**

*Key words:* transcription/regulation/steroid hormones/microinjection/primary cultured cells

## Introduction

The chicken oviduct and liver provide an attractive model system to study how the differentiated specialized eukaryotic cell expresses particular genes under specific conditions. In chicken oviduct tubular gland cells, transcription of the two egg-white proteins conalbumin (or ovotransferrin) and ovalbumin is regulated by the steroid hormones: oestrogens, progestins, glucocorticoids and androgens (Palmiter, 1972; Schimke *et al.*, 1975; Rosen and O'Malley, 1975 and refs therein; Lee *et al.*, 1978). In the absence of steroid hormones, there is a basal level of transcription of the conalbumin gene, whereas transcription of the ovalbumin gene is undetectable (Palmiter, 1975; Mulvihill and Palmiter, 1977; O'Malley *et al.*, 1979; McKnight and Palmiter, 1979; Hynes *et al.*, 1979; Chambon *et al.*, 1979; Hager *et al.*, 1980; LeMeur *et al.*, 1981). In chicken parenchymal liver cells, transcription of the conalbumin gene is regulated by serum iron levels (McKnight *et al.*, 1980a) and is much less sensitive to steroid hormones than in the oviduct (Lee *et al.*, 1978; McKnight *et al.*, 1980b). In contrast, the ovalbumin gene is never expressed in these cells, despite the presence of functional oestrogen receptor molecules which allow oestrogen induction of the vitellogenin II gene transcription (Deeley *et al.*, 1977; Jost *et al.*, 1978; Wilks *et al.*, 1981, 1982 and refs therein). Conversely, the vitellogenin gene is not expressed in the chicken oviduct tubular gland cells.

Clearly, the mere presence or absence of steroid hormone receptors is insufficient to explain how gene expression is controlled in one specialized cell and not in another. There are no chicken liver or oviduct permanent cell lines with functional receptors in which the molecular mechanism of this regulation could be studied. In addition, transfection of primary cultured hepatocytes and tubular gland cells by the calcium phosphate or DEAE-dextran techniques is inefficient (our unpublished results). Thus we have microinjected recombinant plasmids containing chicken conalbumin and ovalbumin promoter regions linked to an SV40 T-antigen reporter gene into primary cultured hepatocytes and oviduct tubular gland cells. T-antigen synthesis was monitored *in situ* by indirect immunofluorescence and used to determine the function of both promoters. A similar approach has been used by Renkavitz *et al.* (1982, 1984) to analyse activation of transcription from the chicken lysozyme gene promoter by progestins and glucocorticoids in primary cultured oviduct tubular gland cells.

We show here that the ovalbumin and conalbumin promoter regions do not function in chicken fibroblasts, kidney cells or in a variety of non-chicken cells, irrespective of the presence of steroid hormone receptors. In contrast, these promoter regions are active in primary cultured chicken embryonic hepatocytes and oviduct tubular gland cells, suggesting that cell-specific transcription factor(s) are present in these cells. In embryonic hepatocytes, ovalbumin and ovotransferrin promoter activity is insensitive to steroid hormones. However, in primary cultured oviduct tubular gland cells, the ovalbumin promoter and, to a lesser extent, the conalbumin promoter are activated by steroid hormones. It appears, therefore, that most of the controls exerted on the activity of ovalbumin and conalbumin promoters in the whole animal can be reproduced *in vitro* by microinjection of chimeric genes into primary cultured cells. There is, however, a striking difference between the two systems; the microinjected ovalbumin promoter is active in cultured embryonic hepatocytes, whereas the ovalbumin gene is never expressed in chicken liver.

## Results

### *A microinjection assay for conalbumin and ovalbumin promoter activity*

It has been previously reported that the conalbumin promoter does not function when introduced into non-chicken cells, such as LMtk$^-$ mouse fibroblasts (Wasylyk and Chambon, 1982) or monkey CV1 cells unless it is activated by the SV40 enhancer (Moreau *et al.*, 1981; Wasylyk *et al.*, 1983). Similarly, the ovalbumin gene is neither accurately nor efficiently expressed when transferred *in vitro* into a variety of non-oviduct cells

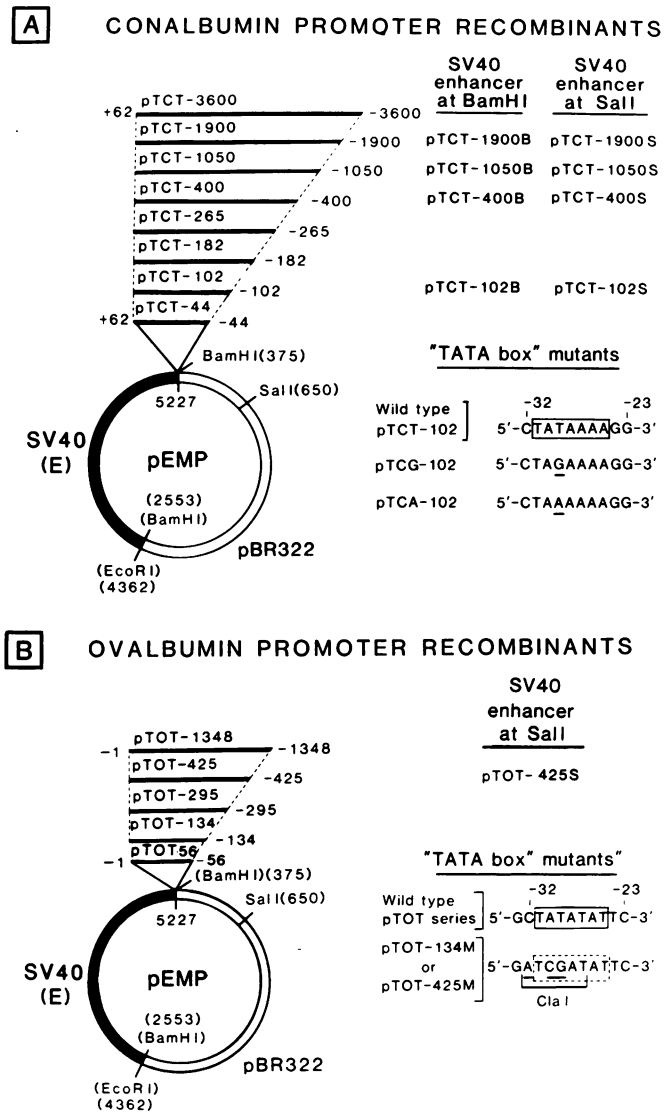


**Fig. 1.** Structure of the conalbumin promoter pTCT (**panel A**) and ovalbumin promoter pTOT (**panel B**) recombinant series. All coordinates of the conalbumin and the ovalbumin fragments are numbered relative to the RNA start site (+1). The thick black line in pEMP represents the SV40 early coding sequence [SV40 (E)] between 5227 and 2553 (coordinates according to the BBB system). The double open lines represent pBR322 sequences. The SV40 enhancer fragment from 270 to 113 was inserted either at the *Bam*HI (375) site of pBR322 to obtain the pTCT-B conalbumin series (panel A, right) or at the *Sal*I site (650) of pBR322 to construct the pTCT-S conalbumin series (panel A, upper right) or the ovalbumin recombinants pTOT-425S (panel B, upper right). Sequences of the mutated TATA box of both conalbumin and ovalbumin recombinants are indicated in the lower right parts of panels A and B respectively. For further details, see Materials and methods.

(Breathnach *et al.*, 1980). To investigate whether the conalbumin promoter inactivity was due to the absence of specific transcription factor(s) in these cells, we used the DEAE-dextran transfection technique to introduce a series of conalbumin promoter recombinants (the pTCT series, Figure 1A) into primary cultured hepatocytes from 18-day-old chicken embryos that synthesize conalbumin mRNA but not ovalbumin mRNA (A.Krust and A.Dierich, unpublished results). Additional recombinants containing the SV40 enhancer element inserted either directly upstream from the conalbumin promoter region (pTCT-B series) or further upstream at the pBR322 *Sal*I site (pTCT-S series) were constructed (see Figure 1A and Materials and methods). Synthesis of T-antigen from the SV40 early region was monitored by indirect immunofluorescence (Moreau *et al.*, 1981) 48 h after transfection into CV1 cells and chicken hepatocytes. The plasmid pSV1 (Benoist and Chambon, 1980), containing the whole SV40 early region including its promoter (which functions in most cell types), was transfected in parallel. The conalbumin promoter region (pTCT series) was inactive in monkey CV1 cells irrespective of its length, unless the SV40 enhancer was present (data not shown). A similar stimulatory effect of the SV40 enhancer was obtained in chicken embryonic hepatocytes when the pTCT-B and pTCT-S series were compared with the pTCT series. Nevertheless the pTCT-B and pTCT-S recombinants were expressed more efficiently in chicken hepatocytes than in monkey CV1 cells. The control SV40 wild-type recombinant, pSV1, was expressed at the same level in both monkey CV1 and chicken liver cells (data not shown).

From these results, we concluded that chicken hepatocytes may contain some factor(s) which activate the conalbumin promoter and are absent in monkey CV1 cells. However, because of the low efficiency of the DNA transfer, the presence of the SV40 enhancer was necessary to reveal this cell specificity. Similarly, the ovalbumin promoter region (pTOT series, see Figure 1B) was inactive in all transfected cell types (including primary cultured oviduct tubular gland cells) unless the SV40 enhancer was present in the recombinants (data not shown). Since all attempts to modify the DEAE-dextran technique or to use the calcium phosphate method failed to improve the DNA transfer efficiency, we decided to study further the cell-specific function of the conalbumin and ovalbumin promoters by direct microinjection of the recombinants into the cell nuclei. This technique has been used successfully to estimate the activity of weak promoter regions, when quantitative S1 nuclease analysis of the RNA synthesized after DNA transfection was not possible unless the SV40 enhancer was added (Moreau *et al.*, 1981; Hen *et al.*, 1982). We have already discussed the validity of the microinjection immunofluorescence assay to estimate the activity of a promoter linked to the T-antigen coding sequence (Moreau *et al.*, 1981; Wasylyk and Chambon, 1982). It is, however, important to stress that comparisons of the results obtained for a given set of recombinants, using either the microinjection immunofluorescence assay or quantitative S1 nuclease analysis of the synthesized RNA after calcium phosphate transfection, have shown that the relationship between the relative number of fluorescent nuclei and the relative promoter efficiency as determined by RNA analysis is non-linear (Hen *et al.*, 1982; Wasylyk *et al.*, 1983). Since the variation in fluorescence intensity could not be measured accurately, the microinjection assay, which scores only the number of fluorescent nuclei, rapidly reaches a saturation level as the activity of the promoter increases. Large decreases in its efficiency are accompanied by very significant decreases in the number of immunofluorescent nuclei, whereas increases of the promoter efficiency above a certain level are reflected by only small increases in this number. Since it was not feasible to routinely microinject different amounts of DNA for each recombinant or culture conditions, we have chosen to microinject ~100 or 200 copies of recombinant DNA, which was usually found to result in the largest variations using different cell types and promoter regions.

*The conalbumin and ovalbumin promoter regions are active in primary cultured chicken embryonic hepatocytes and oviduct tubular gland cells, but not in other cell types*

The pTCT conalbumin and pTOT ovalbumin promoter series were microinjected into primary cultured chicken embryonic

**Table I.** Expression of conalbumin and ovalbumin promoter chimeric recombinants after microinjection into nuclei of primary cultured chicken embryonic and adult heptatocytes maintained in synthetic medium without steroid hormones

| Chimeric recombinants | Immunofluorescent cells (in % of pSV1) | | |
|---|---|---|---|
| | Embryonic cells | | Adult cells |
| | Condition A | Condition B | Condition A |
| Conalbumin promoter | | | |
| pTCT-44 | 6[ 5–8 ] (3) | 8[ 5–11](3) | n.d. |
| pTCT-102 | 28[21–40](10) | 51[40–68](3) | 85[73–98] (2) |
| pTCT-182 | 22[18–26] (3) | 30[20–40](4) | n.d. |
| pTCT-265 | 43[35–52] (6) | 32[26–42](5) | n.d. |
| pTCT-400 | 38[30–46] (6) | 35[30–43](4) | 94[54–126](6) |
| pTCT-1050 | 57[48–65](12) | 60[50–75](6) | n.d. |
| pTCT-1900 | 45[35–46] (4) | 37[26–59](4) | n.d. |
| pTCT-3600 | 17[15–18] (3) | 8[ 7–9 ](3) | n.d. |
| pTCT-1050 + {Tamoxifen ($10^{-6}$ M) RU486 (2 × $10^{-6}$ M) | 50[45–62] (3) | n.d. | n.d. |
| Ovalbumin promoter | | | |
| pTOT-56 | 23[16–42] (5) | n.d. | 4 [0–12] (9) |
| pTOT-134 | 37[22–48] (4) | 36[31–40](3) | 0 (3) |
| pTOT-295 | 27[22–36] (3) | 38[29–43](3) | n.d. |
| pTOT-425 | 34[23–43] (3) | 40[29–50](3) | 0 (10) |
| pTOT-1348 | 37[28–44] (3) | 39[28–52](4) | n.d. |
| pTOT-425 + {Tamoxifen ($10^{-6}$ M) RU486 (2 × $10^{-6}$ M | 35[24–42] (3) | n.d. | n.d. |

Condition A: ~100 copies of DNA were microinjected per nuclei, and the T-antigen positive nuclei scored 24 h after microinjection. Condition B: ~200 copies of DNA were microinjected and the T-antigen-positive nuclei were scored 6 h after microinjection. The number of T-antigen positive hepatocyte nuclei was expressed in % relative to those obtained with the SV40 wild-type recombinant pSV1. In all cases, ~70% of the cells microinjected with pSV1 were T-antigen positive. In each case the average value of several independent experiments (numbers in parentheses) is underlined and the extreme values are indicated within square brackets. When indicated, Tamoxifen and RU486 were added immediately after DNA microinjection. For further details see Materials and methods. N.d.: not determined.

hepatocytes (Table I) and oviduct tubular gland cells (Table II). Very similar results were obtained whether the nuclei were microinjected with ~200 recombinant copies and scored for T-antigen synthesis after 6 h (condition B), or microinjected with ~100 recombinant copies and scored after 24 h (condition A).

All conalbumin and ovalbumin promoter recombinants were expressed in microinjected primary cultured embryonic hepatocytes maintained in a synthetic medium in the absence of steroid hormones (Table I). Addition of steroid hormones (oestradiol $10^{-8}$ M, progesterone $10^{-9}$ M or dexamethasone $10^{-7}$ M) to the synthetic medium, or culturing the cells in a medium containing 10% foetal calf serum (which contains steroid hormones, see below) did not stimulate the activity of the promoters (results not shown). There was a significant increase in the activity of the conalbumin promoter region when its length was increased from 44 to 102 bp, and a significant decrease with a 3600 bp-long 5′-flanking region (pTCT-3600). Note that pTCT-44 which contains no conalbumin gene sequence upstream from the TATA box was specifically expressed in hepatocytes (see below). Augmenting the length of the 5′-flanking region from 56 to 1348 bp did not affect markedly the efficiency of the ovalbumin promoter region. Surprisingly, the ovalbumin gene promoter was active in embryonic liver cells, although this gene is not expressed in embryonic and adult chicken liver even after steroid hormone administration (McKnight *et al.*, 1980a,b; our unpublished results). However, the ovalbumin promoter was inactive in primary cultured liver cells prepared from the 3-week-old chicken and maintained in 10% foetal calf serum (Table I).

The pTCT and pTOT recombinants were also expressed following microinjection into chicken oviduct tubular gland cells maintained in a medium containing 10% foetal calf serum (Table II). Such a medium contains oestradiol, progesterone and glucocorticoids at concentrations of ~$10^{-11}$ to $10^{-10}$ M (data not shown). In contrast to primary cultured hepatocytes, primary cultured oviduct tubular gland cells could not be maintained in serum-free media (our unpublished observations). In general, the relative activity of all the ovalbumin promoter recombinants appears to be higher in tubular gland cells than in hepatocytes (compare pTOT recombinants in Tables I and II). Increasing the length of the ovalbumin 5′-flanking sequence did not increase ovalbumin promoter activity. Note that 56 bp of the 5′-flanking ovalbumin promoter sequence were sufficient to promote specific and apparently efficient expression (see below). Again, we observed clear variations in the relative activity of the conalbumin promoter region depending on its length, and a marked increase in activity between pTCT-44 and pTCT-102. As in hepatocytes (Table I) recombinant pTCT-44, which is truncated immediately upstream of the TATA box, was specifically expressed in oviduct tubular gland cells (see below). There was a significant decrease between the relative promoter activies of pTCT-102 and

pTCT-265, whereas the relative promoter activities of pTCT-400 and pTCT-1050 were very high, the number of immunofluorescent tubular gland cells reaching that obtained after microinjecting pSV1, the wild-type SV40 recombinant.

**Table II.** Expression of conalbumin and ovalbumin promoter chimeric recombinants after microinjection into nuclei of primary cultured chicken oviduct tubular gland cells maintained in a medium containing foetal calf serum

| Chimeric recombinants | Immunofluorescent cells (% of pSV1) | |
|---|---|---|
| | Condition A | Condition B |
| Conalbumin promoter | | |
| pTCT-44 | 16[12–20] (4) | 29[21–36] (4) |
| pTCT-102 | 50[38–62] (6) | 71[48–98] (6) |
| pTCT-182 | 59[45–80] (4) | 38[34–46] (4) |
| pTCT-265 | 26[25–34](10) | 32[30–36] (3) |
| pTCT-400 | 70[48–96] (6) | 84[72–97] (5) |
| pTCT-1050 | 71[54–95] (6) | 108[88–136](5) |
| pTCT-1900 | 31[30–52] (4) | 52[40–67] (6) |
| pTCT-3600 | 25[20–34] (5) | 21[13–35] (6) |
| + {Tamoxifen ($10^{-6}$ M) RU486 ($2 \times 10^{-6}$ M) | | |
| pTCT-102 | 49[34–58] (3) | n.d. |
| pTCT-400 | 42[40–44] (4) | n.d. |
| Ovalbumin promoter | | |
| pTOT-56 | 75[68–85] (5) | n.d. |
| pTOT-134 | 58[55–64[ (4) | 70[68–71[ (3) |
| pTOT-295 | 53[46–61] (4) | 55[42–61] (6) |
| pTOT-425 | 59[39–73] (5) | 48[38–57] (5) |
| pTOT-1348 | 46[41–54] (6) | 28[26–32] (5) |
| pTOT-425 + {Tamoxifen ($10^{-6}$ M) RU486($2 \times 10^{-6}$ M) | 8[ 0–16) (8) | n.d. |

Conditions A and B were as described in footnote to Table I. The number of T-antigen positive oviduct cell nuclei was scored and the results are expressed as described in footnote to Table I. In all cases 50–60% of the cells microinjected with pSV1 were T-antigen positive. When indicated, Tamoxifen and RU486 were added immediately after DNA microinjection. N.d.: not determined. For further details see Materials and methods.

Approximately 200 copies of the pTCT and pTOT recombinants were also microinjected into the nuclei of cell lines from monkey (CV1) mouse (LMtk$^-$) and human [HeLa and MCF-7, the latter containing oestrogen and progestin receptors (Horwitz and McGuire, 1978; Westley and Rochefort, 1980)], as well as into nuclei of primary cultured mouse hepatocytes, chicken fibroblasts and kidney cells. The conalbumin and ovalbumin promoter regions did not function in any of the cells tested, with the possible exception of MCF-7 cells in which the ovalbumin promoter regions present in pTOT-134, pTOT-295 and pTOT-425 were very weakly active (~2% of pSV1, data not shown). It was shown that these two promoter regions can be activated and function in these cells by microinjecting the SV40 enhancer-containing pTCT-102B and pTOT-425S recombinants which were efficiently expressed, whereas the recombinant pEMPB which contains only the SV40 enhancer and the T-antigen coding sequence (Moreau *et al.*, 1981) was weakly expressed (results not shown). Furthermore, significant T-antigen synthesis was observed in the same cells when the ovalbumin or conalbumin promoter regions were replaced by either the adenovirus-2 major late (Ad2ML) promoter region from +33 to −34 (recombinant pSVA34) or +33 to −677 (recombinant pSVA677, Hen *et al.*, 1982), or by the adult chicken β-globin promoter region from +43 to −600 (recombinant pADG) (Table III), indicating that these cells were not intrinsically unable to initiate transcription from heterologous promoter elements.

It is noteworthy that the ovalbumin and conalbumin promoter chimeric recombinants were not active when microinjected into the fibroblasts also present in the primary cultures of oviduct cells. This is illustrated in Figure 2 which shows selected fields of primary cultured oviduct tubular gland cells (panels b, c and d) or fibroblasts (panels e and f) which were microinjected with either pTOT-56 (panel b), D10 (panels c and e) or pTOT-56 and D10 (panels d and f). D10 is an SV40 recombinant (Kalderon *et al.*, 1984) bearing a T-antigen point mutation, which prevents its nuclear translocation. Although the cytoplasm of both tubular gland cells (panels c and d) and fibroblasts (panels e and f) were immunofluorescent after microinjection of D10, only the nuclei of tubular gland cells (panel d), and not those of fibroblasts (panel f), were T-antigen positive when pTOT-56 was co-microinjected.

### *The expression of the pTOT and pTCT recombinants is under the control of proper promoter elements*

No T-antigen expression was ever observed in any of the cells microinjected with the recombinant pEMP (Figure 1, see Moreau

**Table III.** Expression of the adenovirus-2 major late (Ad2ML, pSVA34 and pSVA677) and chicken β-globin (pADG) promoter chimeric recombinants after microinjection into the nuclei of various cell types

| Chimeric recombinants | Immunofluorescent cells (in % of pSV1) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Cell lines | | | Primary cultured chicken cells | | | |
| | HeLa | CV1 | MCF-7 | Hepatocytes | Oviduct | Fibroblasts | Kidney |
| pTCT-44 | 0 | 0 | 0 | 6 | 16 | 0 | 0 |
| pTOT-56 | 0 | 0 | 0 | 23 | 75 | 0 | 2 |
| AD2MLP promoter | | | | | | | |
| pSVA34 | 7 | 6 | n.d. | 23 | 18 | 19 | n.d. |
| pSVA677 | 20 | 43 | 37 | 25 | 17 | 19 | n.d. |
| Chicken β-globin promoter (pADG) | 14 | n.d. | n.d. | 47 | 45 | 24 | 40 |

Approximately 100 copies of DNA were microinjected per nucleus of each cell type. The number of T-antigen-positive nuclei was determined as described in footnote to Table I. After microinjection with pSV1, 40–70% of nuclei of each cell type were T-antigen positive. In each case, the numbers are the average values of three independent experiments. For further details see Materials and methods. N.d.: not determined.

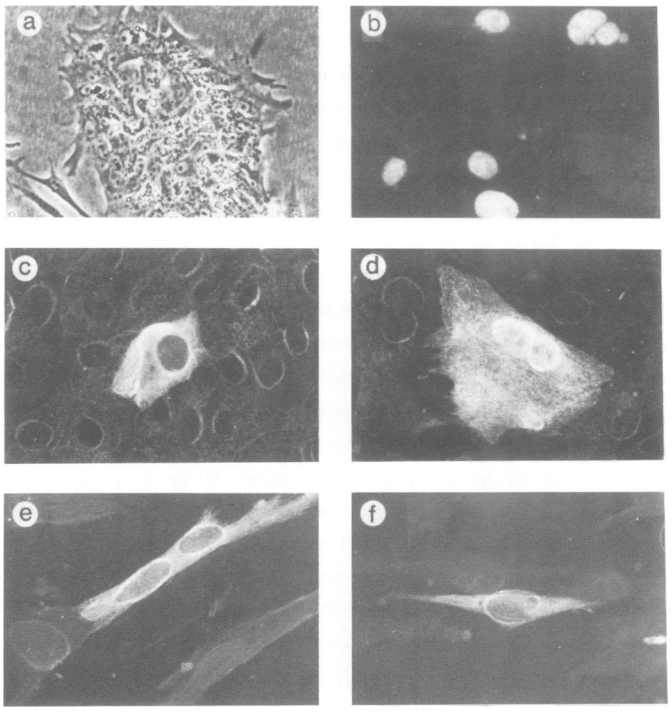


**Fig. 2.** Expression of microinjected ovalbumin promoter chimeric recombinants in chicken oviduct tubular gland cells and fibroblasts in primary culture: (**a**) phase contrast photomicrograph (× 125) of chicken oviduct tubular gland cells 72 h after plating; (**b–f**) chicken oviduct tubular gland cells and fibroblasts (× 500) stained by indirect immunofluorescence using T-antigen monoclonal antibodies. (**b–d**). Oviduct tubular gland cell nuclei microinjected with pTOT-56 (**b**), D10 (**c**), and a mixture of pTOT-56 and D10 (**d**). (**e–f**). Chicken fibroblasts microinjected with D10 (**e**) and a mixture of pTOT-56 and D10 (**f**). pTOT-56 and D10 were microinjected at 200 copies and 20 copies of DNA per cell nucleus respectively.

**Table IV.** Effect of TATA box mutations on the activity of the conalbumin and ovalbumin promoter after microinjection in primary cultured chicken embryonic hepatocytes and oviduct tubular gland cells

| Chimeric recombinants | Immunofluorescent cells (in % of pSV1) | |
|---|---|---|
| | Oviduct tubular gland cells (A) | Hepatocytes (B) |
| Conalbumin promoter | | |
| pTCT-102 | 59[44–75](2) | 55[47–63](2) |
| pTCA-102 | 15[12–19](2) | 15[15–16](2) |
| pTCG-102 | 16[14–17](2) | 18[12–23](2) |
| Ovalbumin promoter | | |
| pTOT-134 | 58[55–64](4) | n.d. |
| pTOT-134M | 17[14–21](3) | n.d. |
| pTOT-425 | 59[39–73](5) | 48[38–57](5) |
| pTOT-425M | 13[ 7–18](8) | 5[ 3– 8](4) |

The sequences of the mutated TATA box of the conalbumin and ovalbumin promoter recombinants are given in panels A and B of Figure 1 respectively. Approximately 100 copies of DNA were microinjected per nucleus. The number of T-antigen-positive liver and oviduct cell nuclei was scored 24 h later and the results are expressed as in Table I. (A) Depending on the experiment, 50–64% of the cells microinjected with pSV1 were T-antigen postive. (B) Depending on the experiment, 50–70% of the microinjected cells with pSV1 were T-antigen positive. N.d.: not determined.

*et al.*, 1981), which contains only the T-antigen coding sequence but neither ovalbumin nor conalbumin 5′-flanking regions. Although this observation indicates that these regions are necessary for transcription of the T-antigen-coding sequence, they do not exclude the possibility that transcription could be initiated at non-physiological sites in a cell-specific manner. Since the amount of transcribed RNA, which can be recovered from microinjected cells, is too low to permit S1 nuclease mapping of its 5′ end, we have used ovalbumin and conalbumin recombinants mutated in their TATA box to prove that T-antigen synthesis by the pTOT and pTCT recombinants is under the control of the proper promoter elements. Mutations in the TATA box of the conalbumin promoter, pTCA-102 and pTCG-102 (Figure 1A), decreased its activity by 3- to 4-fold, both in oviduct cells and in hepatocytes (Table IV). These decreases correlate with those previously obtained in non-chicken cells with recombinants containing the SV40 enhancer and wild-type or TATA box-mutated conalbumin promoter regions (Wasylyk and Chambon, 1981; Wasylyk *et al.*, 1983). Similar results were obtained both in oviduct cells and hepatocytes for the two TATA box-mutated ovalbumin recombinants pTOT-134M and pTOT-425M (Figure 1B and Table IV). These results support the conclusion that transcription from the microinjected ovalbumin and conalbumin recombinants was controlled by the physiologically relevant promoter elements.

*The activity of the ovalbumin and conalbumin promoter regions is not controlled by steroid hormones in hepatocytes*

As a first step towards the characterization of the ovalbumin and conalbumin promoter sequences which are responsible for steroid hormone induction, we studied the effect of Tamoxifen and RU486 on the activity of the most highly expressed pTOT (pTOT-425) and pTCT (pTCT-400 or pTCT-1050) recombinants in primary cultured embryonic hepatocytes and oviduct tubular gland cells. Tamoxifen is a well-characterized antioestrogen (Sutherland *et al.*, 1977) and RU486 exhibits both anti-glucocorticoid and anti-progestin activity in mammals (Philibert *et al.*, 1981), but is a specific glucocorticoid antagonist in chicken (Groyer *et al.*, 1985; M.Govindan, personal communication). It is important to note that none of these antagonists had any effect on the level of expression of the wild-type SV40 early region recombinant pSV1 in any of the cell types examined (data not shown).

As expected from the absence of steroid hormones in the synthetic culture medium, the addition of both Tamoxifen and RU486 to primary cultured embryonic hepatocytes had no effect on the activity of the conalbumin or ovalbumin promoter region present in pTCT-1050 or pTOT-425 (Table I). Similarly, addition of oestradiol ($10^{-8}$ M) to primary adult chicken hepatocyte cultures in the presence of serum did not stimulate the expression of pTCT-400 (results not shown), or of pTOT-425, which is inactive in these cells. In marked contrast, in oviduct tubular gland cells, the ovalbumin promoter activity was drastically decreased by the addition of the antagonists to the culture medium (Table II). The activity of the conalbumin recombinant pTCT-400 was also significantly decreased by the addition of the anti-oestrogen and anti-glucocorticoid to the oviduct cells in primary culture, whereas the expression of pTCT-102 was not affected by these antagonists (Table II). However, the conalbumin promoter region present in pTCT-400 retained a high level of 'constitutive' activity under conditions where the activity of the corresponding ovalbumin promoter region was blocked almost totally by the steroid hormone antagonists (Table II).

## Discussion

By microinjection of chimeric recombinants containing the conalbumin (the pTCT series) and ovalbumin (the pTOT series) promoter regions and the SV40 T-antigen coding sequence into the nuclei of different cell types in culture, we show here that the 5′-flanking sequences of both chicken genes are involved in cell-specific expression. The pTCT recombinants are expressed specifically in primary cultured liver cells (embryonic or adult) and in oviduct tubular gland cells, in both of which the conalbumin gene is known to be expressed specifically *in vivo*. In contrast, the microinjected ovalbumin recombinants are expressed specifically in both primary cultured embryonic hepatocytes and oviduct tubular gland cells, although the endogenous ovalbumin gene is expressed only in the oviduct. It is noteworthy that the ovalbumin promoter recombinants which exhibit this unforeseen activity in embryonic liver cells are inactive in liver cells of 3-week-old chicks. The lack of expression of the recombinant pEMP (Moreau *et al.*, 1981) which contains the coding region of the SV40 T-antigen, but no conalbumin or ovalbumin promoter sequences, and the 3- to 4-fold decrease in expression in liver and in oviduct cells of the four recombinants with mutated TATA boxes (pTCA-102, pTCG-102, pTOT-134M and pTOT-425M) support the conclusion that cell-specific transcription of the microinjected pTCT and pTOT recombinants is controlled by physiologically relevant promoter elements, in particular the TATA box. Our results do not correlate with those obtained by Lai *et al.* (1983) who found that an ovalbumin gene recombinant is active when stably integrated in human MCF-7 cells, but in agreement with those of Renkawitz *et al.* (1982) who showed that the promoter of another egg-white protein gene, the lysozyme gene, is active when microinjected into nuclei of primary cultured oviduct cells, but not in other cells. Matthias *et al.* (1982) have also observed that the chicken lysozyme gene promoter is inactive when transfected in MCF-7 cells unless the SV40 enhancer is present in *cis* in the recombinant. Furthermore, the presence of steroid hormones had no effect on the activity of the lysozyme promoter.

The pTCT and pTOT series are expressed with a higher efficiency in oviduct tubular gland cells than in embryonic hepatocytes. This observation could reflect either a lower efficiency of both promoter regions in hepatocytes or cell−cell variations in the ability of cultured hepatocytes to use the conalbumin and ovalbumin promoters. The latter possibility is the most likely, since increasing further the number of microinjected recombinant copies did not result in a significant increase in the number of immunofluorescent nuclei (although all of the cells microinjected with the wild-type SV40 recombinant pSV1 had fluorescent nuclei; data not shown). Variations in the ability of the cultured oviduct tubular gland cells to use the ovalbumin promoter may also explain why we have never observed 100% of fluorescent nuclei after microinjection of the pTOT series (Table II and results not shown).

The inactivity of pTCT and pTOT recombinants in monkey (CV1 cells) mouse (LMtk$^-$ fibroblasts, primary cultured liver cells) and human (HeLa, MCF-7) cells, as well as in chicken embryonic fibroblasts and adult kidney cells in primary culture, suggests the existence of cell-specific positive transcription factor(s) which are restricted to embryonic liver and adult oviduct cells. However, this cell-specific factor activity may also be species-specific, since the conalbumin or ovalbumin recombinants were inactive in primary cultured mouse hepatocytes. In addition, some of the putative liver factor(s) appear to be specific to certain stages of differentiation, since expression of the ovalbumin promoter was observed in embryonic but not in adult primary cultured cells. Moreover, oviduct cells may be richer in these factor(s), since pTOT-56 appears to be more efficiently expressed in oviduct tubular gland cells than in embryonic hepatocytes. The alternative possibility, that a negative control mechanism is responsible for the inactivity of the conalbumin and ovalbumin promoters in cells other than hepatocytes and oviduct tubular gland cells, appears very unlikely, since this would require that negative factor(s) acting specifically on these promoters are present in all of these cells, irrespective of their tissue and/or species origins.

Both the conalbumin (−44 to +62) and adenovirus-2 major late (−34 to +33) promoter segments can form stable preinitiation complexes with transcription factor(s) present in a HeLa S100 whole cell extract (Davison *et al.*, 1982, 1983). Although the TATA box sequence of both promoter regions is very similar (Benoist *et al.*, 1980), and the Ad2MLP recombinant pSVA34 is ubiquitously expressed (Table III), the activity of the conalbumin promoter recombinant pTCT-44 is unexpectedly cell-specific *in vivo*. Similarly, the first 56 bp of the 5′-flanking region of the ovalbumin gene appear to be sufficient to ensure cell-specific promoter activity. These obervations suggest strongly the existence of either promoter-specific TATA box factors or of additional chicken liver and oviduct cell-specific factors which could interact with the −44 to +62 conalbumin and −1 to −56 ovalbumin promoter sequences, and be required for initiation of transcription *in vivo*. In addition, these putative factors must be different since only the conalbumin promoter remains active in adult hepatocytes. All of these observations are in contrast with all previous reports which have indicated that cell-specific expression is conferred by promoter elements located either further upstream or further downstream to the TATA box region (see Wasylyk, 1986 for refs).

Although the first 44 bp of the 5′-flanking region of conalbumin gene are sufficient to promote cell-specific expression, additional upstream sequences appear to be required for efficient expression in both embryonic hepatocytes and oviduct cells, since pTCT-102 is better expressed than pTCT-44 (Tables I and II). The 'CAAT' box sequence (for reviews see Wasylyl, 1986; McKnight and Tjian, 1986) which is present in the conalbumin promoter region around −80 may be involved in this increased expression. Surprisingly, a similar increase is not observed between the ovalbumin promoter recombinants pTOT-56 and pTOT-134, although it has recently been shown that the 'CAAT' box, which is present around −80 in the ovalbumin promoter region, is important for the activity of this promoter in HeLa cells (Pastorcic *et al.*, 1986). However, since the number of fluorescent nuclei is not linearily proportional to the relative promoter efficiency as measured from quantitative RNA determination (see Results), the assay used in the present study may preclude the observation of the effect of the ovalbumin 'CAAT' box region.

Additional sequences and factor(s) appear to be involved in the activity of the conalbumin promoter region, since increasing the length of the 5′-flanking region in the pTCT series to −400 and −1050 results in an increase of expression, especially in oviduct cells (Table II). Some of this stimulation may be steroid hormone-related, since addition of an anti-oestrogen (Tamoxifen) and an anti-glucocorticoid (RU486) results in a decrease of expression down to the level of pTCT-102 whose activity is insensitive to the antagonists (Table II). In contrast, no increase in the expression of the pTOT series could be observed with recom-

binants bearing the ovalbumin 5′-flanking regions of increasing lengths. Furthermore, the expression of pTOT-425 is abolished almost completely in the oviduct cells in the presence of both Tamoxifen and RU486, whereas no change is observed in hepatocytes maintained in serum-free medium. These observations are in agreement with the *in vivo* stimulatory effect of steroid hormones on the transcription rate of the conalbumin and ovalbumin genes in oviduct tubular gland cells, which is known to be much more dramatic for the ovalbumin than for the conalbumin gene (McKnight and Palmiter, 1979). Further studies on the effect of steroid hormones on the ovalbumin promoter activity are reported in the accompanying paper (Gaub *et al.*, 1987).

In conclusion, the present studies show that the *in vivo* cell-specific activity of the ovalbumin and conalbumin promoters can also be observed by microinjecting chimeric recombinants containing these promoters in primary cultured adult hepatocytes and oviduct tubular gland cells. Unexpectedly, proximal promoter sequences located close to the TATA box are sufficient to confer cell-specific expression. In addition, an ovalbumin promoter, microinjected in embryonic (but not in adult) hepatocytes, escapes the regulation imposed on the corresponding endogenous inactive gene. It appears that the non-expression of the ovalbumin gene in the liver of the chicken embryo is not due to the lack of a cell-specific transcription factor(s), but may be due to a negative mechanism related for instance to the permanent 'closing' of the gene during development [e.g. DNA methylation and/or closed chromatin conformation as indicated by the lack of chromatin DNase I sensitivity and hypersensitivity over the ovalbumin gene promoter region in the chicken liver (Kaye *et al.*, 1986)], which can be bypassed by microinjection of plasmid recombinants. This ovalbumin gene cell-specific transcription factor is apparently absent (or inactive) in adult hepatocytes, as well as in other chicken cells (fibroblasts, primary cultured kidney cells).

## Materials and methods

*Cell lines*

HeLa cells (gift from W.Schaffner) were cultivated as monolayer in Dulbecco's modified Eagle's medium (DME) supplemented with 2.5% foetal calf serum (FCS), 2.5% calf serum (CS), penicillin (60 mg/l) and streptomycin (100 mg/l). MCF-7 cells were grown as monolayer in DME containing FCS (10%), porcine insulin (0.6 mg/l) and antibiotics. LMtk$^-$ mouse cells were cultivated in DME plus 10% FCS and antibiotics. CV1 (monkey kidney) cells were grown in Eagle's medium supplemented with 10% FCS.

*Primary cultured cells*

Chick embryo liver cells were prepared from 18-day-old chicken embryos as described by Fischer and Marks (1976) with minor modifications. After the collagenase treatment, the cells were filtered through a nylon sieve (48-$\mu$m mesh) before centrifugation. The plated cells, in Petri dishes (6-cm diameter, $\sim 2 \times 10^6$ cells/dish) were incubated for several hours in Waymouth medium (MD705/1) supplemented with 5% CS to favour their attachment to the dish, and then maintained in Waymouth serum-free medium containing insulin (1 mg/ml) and antibiotics. 'Adult' liver cells were similarly prepared from livers of 3-week-old oestradiol-treated chickens (see below), but were maintained in culture in Waymouth medium containing 10% FCS supplemented with insulin (1 mg/ml) and antibiotics.

New-born mouse liver cells were prepared as the chicken embryo liver cells, but were maintained in DME supplemented with 10% FCS, insulin (1 mg/l) and antibiotics.

Chicken embryonic fibroblasts and kidney (3-week-old chicken) cells were prepared according to Solomon (1976).

Primary cultured oviduct cells were prepared as follows. Oviduct magna were removed from 3-week-old chickens (Warren I.S.A. Brown Strain) stimulated for 10 days with diethylstilbestrol (DES; 15 mg) pellets. The oviducts were washed three times in sterile phosphate saline buffer (PBS), minced with scissors in PBS, and trypsinized gently (0.5% trypsin in PBS−EDTA 1 mM) for 8 min at 37°C. The supernatant was decanted and made 5% in FCS to inhibit trypsin action. The trypsinization of the pellets was repeated three to four times. All supernatants were pooled and filtered through gauze. The filtered cells were pelleted, resuspended in fresh DME medium supplemented with 10% FCS, 5% bactotryptose phosphate, 1 mg/l insulin, and cultivated in Petri dishes at 37°C in 95% air 5% $CO_2$ ($\sim 10^6$ cells/6-cm dish). In some experiments, FCS was treated with dextran-coated charcoal overnight at 4°C, to remove endogenous steroids. In this case, dishes were precoated with rat tail collagen before plating. The oviduct tubular gland cells were usually microinjected 3−4 days after plating.

*Construction of chimeric recombinants*

The conalbumin pTCT recombinant series was generated by inserting (blunt-end ligation) different fragments of the conalbumin gene 'Eco b' fragment (Cochet *et al.*, 1979) [from +62 (*Alu*I site) to the more upstream restriction sites *Hha*I, *Bst*NI, *Pst*I, *Bam*HI, *Bgl*II, *Bam*HI and *Eco*RI, for pTCT−44, −182, −265, −400, −1050, −1900 and −3600 respectively] in the repaired unique *Bam*HI site of pEMP (Benoist and Chambon, 1980) (see Figure 1A). pTCT-102, pTCT-102S, and pTCT-102B were described previously as pTCT, pTCB3 and pSVCT respectively, in Moreau *et al.* (1981). In all cases a *Bam*HI restriction site was regenerated at position +62 of the conalbumin gene. The conalbumin fagments are in the same 'transcriptional' orientation as the SV40 T-antigen coding sequences. For the pTCT-S series, the conalbumin sequences from +62 to −400, −1050 and −1900 were cloned in the unique *Bam*HI site of pEMPS [called pEMPB in Moreau *et al.* (1981)] which had the enhancer sequence of SV40 (coordinates 113−270) cloned in the *Sal*I site of pBR322 to yield pTCT-400S, pTCT-1050S and pTCT-1900S, respectively. For the pTCT-B series, the 72-bp repeat sequence of SV40 (113−270) was excised from pHS102 (Benoist and Chambon, 1981) and cloned in partially *Bam*HI-digested pTCT-400, pTCT-1050 and pTCT-1900 to give pTCT-400B, pTCT-1050B and pTCT-1900B respectively. Recombinants up to pTCT-265 were sequenced. For the other conalbumin recombinants, the orientation of the conalbumin promoter with respect to the coding SV40 region was established by restriction enzyme analysis. The recombinants in which the conalbumin TATA box sequence has been mutated have been described previously (Wasylyk *et al.*, 1980; Wasylyk and Chambon, 1981).

The ovalbumin pTOT recombinant series was generated by blunt-end insertion of different restriction fragments prepared from the pBR322-based recombinant containing the *Eco*RI−*Pst*I ovalbumin promoter region fragment (Mulvihill *et al.*, 1982) into the unqiue *Bam*HI site of pEMP (Figure 1B). Insertion of the fragments *Rsa*I−*Ava*II (−1 to −134), *Rsa*I−*Sau*3A (−1 to −295), *Rsa*I−*Rsa*I (−1 to −425) and *Rsa*I−*Pst*I (−1 to −1348) yielded the recombinants pTOT-134, pTOT-295, pTOT-425 and pTOT-1348 respectively. pTOT-56 was obtained by *Bal*31 nuclease digestion of the ovalbumin fragment −1 to −425 subcloned in M13, followed by blunt-end insertion into the unique *Bam*HI site of pEMP. All the ovalbumin recombinants were sequenced throughout the inserted sequence.

The ovalbumin TATA box of pTOT-134 and pTOT-425 was mutated according to Grundström *et al.* (1985) using the synthetic oligonucleotide (3′-GTTAAGTCCTAGCTATAAGGG-5′) which generates a *Cla*I restriction site (5′-ATCGAT-3′) to yield pTOT-134M and pTOT-425M respectively (Figure 1B).

The chicken $\beta$-globin pADG recombinant was constructed by blunt-end ligation of the *Rsa*I (+43)−*Eco*RI ($\sim -610$) fragment of the adult chicken $\beta$-globin gene isolated from p$\beta$1Br15 (a gift from Dr Engel; Dodgson *et al.*, 1979, 1983; Dolan *et al.*, 1983) into the repaired unique *Bam*HI site of pEMP. The orientation of the $\beta$-globin promoter with respect to the SV40 coding region was determined by restriction mapping. The adenovirus-2 major late promoter (Ad2MLP) recombinants pSVA34 and pSVA677 have been described previously (Hen *et al.*, 1982).

*DNA transfer and microinjection*

DNA was transfected into monkey CV1 or primary cultured embryonic hepatocytes using the DEAE-dextran technique (McCutchan *et al.*, 1968), except that 1 $\mu$g of plasmid DNA per 6-cm Petri dish was used, or by the calcium phosphate precipitation technique of Wigler *et al.* (1977). After a 36−48-h incubation, expression of SV40 T-antigen was detected by indirect immunofluorescence using T-antigen antibodies as described previously (Moreau *et al.*, 1981). Five millimeter-diameter circles were marked on each plate under areas of confluent cells. For each recombinant, the results were expressed as a percentage of T-antigen positive cells (cells with fluorescent nuclei) relative to the expression of the SV40 wild-type recombinant pSV1 (Benoist and Chambon, 1980) with which $\sim 1\%$ of the CV1 or liver cell nuclei were fluorescent. All positive cells were taken into account, irrespective of the intensity of the fluorescence.

DNA was microinjected into cell nuclei using the technique of Graessmann *et al.* (1980) as modified by Ansorge (1982). In each experiment, between 60 and 100 nuclei were microinjected (in two places on the Petri dish). The number of DNA copies microinjected per nucleus was estimated according to Graessmann *et al.* (1980). T-antigen positive cells were scored 6 or 24 h after microinjection and the results were expressed relative to pSV1 as described above. For each recombinant, several DNA preparations were tested.

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