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# Organization of the thyroid hormone receptor in the chromatin of C6 glial cells: evidence that changes in receptor levels are not associated with changes in receptor distribution

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The association of [$^{125}$I]T3-receptor complexes with C6 cell chromatin was analyzed after a limited digestion with micrococcal nuclease (MN) or DNase I. Both nucleases solubilized up to 60–70% of receptor and 0.4 M KCl extracted 70% of the non-digested receptor, thus showing that only a residual fraction of receptor is associated with the nuclear matrix. With DNase I the receptor was released 2–3-fold faster than the bulk of chromatin, whereas a preferential release of receptor over total chromatin was not observed with MN. The digestion of receptor with DNase I and MN occurred 14- and 6-fold faster, respectively, than the appearance of PCA-soluble chromatin. Preincubation for 48 h with 4 nM T3 of 2 mM butyrate significantly altered receptor levels but did not change sensitivity to the nucleases. These results suggest that the thyroid hormone receptor is associated with chromatin highly sensitive to nuclease digestion, and that changes in receptor number are not associated with changes in its distribution in chromatin.

Thyroid hormone; Receptor; Chromatin; Micrococcal nuclease; DNase I

## 1. INTRODUCTION

After entering the cells thyroid hormones bind to nuclear receptor proteins. The association of the receptor with chromatin has been investigated by the use of nuclease digestion and differential solubilization [1,2]. Whereas T3 receptors in hepatic nuclei are preferentially associated with 'active' regions of chromatin highly sensitive to DNase I and micrococcal nuclease (MN) [1], in pituitary GH1 cells the bulk of receptor appears to be homogeneously dispersed throughout the chromatin [2]. In both systems MN excises the receptor as an abundant 6.5 S form and as a minor 12.5 S species. This has been interpreted as the 6.5 S form representing receptor associated with internucleosomal linker DNA regions, and the 12.5 S form representing receptor excised in association with mononucleosomes.

Thyroid hormone receptor levels in cells can be influenced by a variety of factors. We have recently demonstrated that in glial C6 cells triiodothyronine (T3) elicits a time- and dose-dependent down-regulation of receptors [3]. In contrast to thyroid hormones, butyrate and other short-chain fatty acids increase receptor levels by a mechanism which could involve hyperacetylation of chromatin proteins [4].

In this study we have examined the organization of thyroid hormone receptor and its accessibility to limited digestion by nucleases in control and T3- or butyrate-treated C6 cells. The results obtained show that the receptors are not evenly distributed in the chromatin of C6 cells, and that alterations in receptor levels are not associated with its distribu-

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*Abbreviations:* T3, 3,5,3'-triiodo-L-thyronine; MN, micrococcal nuclease

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tion between active and inactive regions of chromatin.

## 2. MATERIALS AND METHODS

### 2.1. *C6 cell culture*

C6 cells were grown in monolayers as in [3–5]. To ensure a complete cellular depletion of thyroid hormone 48 h before the beginning of the experiments the growth medium was replaced by fresh medium containing 10% newborn calf serum depleted of thyroid hormone [5].

### 2.2. *[$^{125}$I]T3 labeling and nuclease digestion of nuclei*

The cell monolayers were incubated with 2 mM butyrate or 4 nM [$^{125}$I]T3 for 48 h in thyroid hormone-depleted medium. During the last 2 h control and butyrate-treated cells which were not previously exposed to the hormone received 4 nM [$^{125}$I]T3. This concentration of T3 binds more than 90% of the receptor population [5]. Parallel cultures were incubated with a 1000-fold excess of non-radioactive T3 to determine the non-specific binding which was subtracted from the data. The nuclei were isolated [3–5] and washed with a buffer containing 10 mM Tris-HCl, pH 7.4 at 25°C, 1 mM $CaCl_2$, 10 mM KCl, 1 mM $MgCl_2$, 5 mM 2-mercaptoethanol and 20 U/ml Trasylol. The nuclei were digested with MN (0.25–0.3 U/l $A_{260\,nm}$ U nuclear material) or with DNase I (2–3 U/l $A_{260\,nm}$ U nuclear material) at 0°C as in [2]. After digestion the samples were adjusted to 10 mM EDTA to stop the reaction and centrifuged at 3000 × *g* for 10 min. The [$^{125}$I]T3 associated to the supernatant and the residual nuclear pellet was determined to calculate the % of receptor released during the digestion. The absorbance of both fractions was analyzed at 260 nm to determine the % of total chromatin solubilized by the enzymes, and an aliquot of the supernatant was precipitated with 7% perchloric acid (PCA) to determine the fraction of the solubilized material which has been rendered PCA-soluble. All data are the mean of triplicate samples with less than 5–10% variation and were reproduced at least 3 times with similar results.

### 2.3. *Sedimentation of receptor after micrococcal nuclease digestion of nuclei*

In some experiments the supernatants were centrifuged at

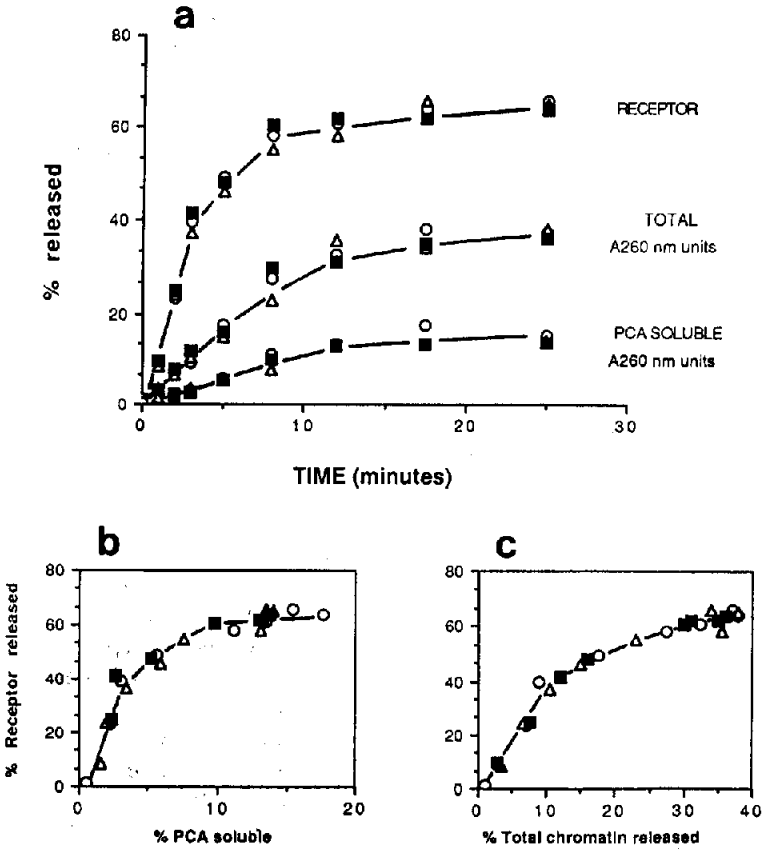


Fig.1. DNase I digestion kinetics and receptor release. Nuclei from control cells (○) or from cells incubated for 48 h with 4 nM T3 (△) or 2 mM butyrate (■) were labeled with [$^{125}$I]T3 and digested with DNase I for the times indicated in panel (a) as described in section 2. (a) Kinetics of digestion, (b) relationship between receptor release and generation of PCA-soluble chromatin, (c) receptor release vs total chromatin solubilized.

4°C in isokinetic sucrose gradients as in [5] and after centrifugation were resolved into approx. 35 fractions. Trichloroacetic acid was added to each fraction to achieve a final concentration of 15% (w/v). The samples were chilled, and after determination of [$^{125}$I]T3, were centrifuged at 3000 × *g* for 30 min. The precipitates were analyzed for DNA by the method of Burton [6].

2.4. *Salt extraction of digested nuclei*

The cells were incubated with 4 nM [$^{125}$I]T3 with or without a 1000-fold excess of unlabeled hormone for 48 h. A group of cells incubated with the same concentrations of [$^{125}$I]T3 for 90 min were used as controls, and additional cultures incubated with 2 mM butyrate for 48 h were also labeled with [$^{125}$I]T3 during the last 90 min. After isolation of nuclei, the nuclear pellets were digested with 6 U DNase I/$A_{260nm}$ U nuclei for 30 min at 4°C. The samples were centrifuged at 3000 × *g* for 10 min and the radioactivity of the pellet and the supernatant determined. The remaining nuclear material was extracted twice with 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 20 U/ml Trasylol containing 0.4 M KCl and after centrifugation the [$^{125}$I]T3 associated to the pellet (crude nuclear matrix) and the supernatant was analyzed.

## 3. RESULTS

Fig.1a shows the kinetics of liberation of [$^{125}$I]T3 receptor complex from chromatin after DNase I treatment. When nuclei were incubated for up to 25 min in the absence of added nuclease less than 3% of chromatin-bound radioactivity was released. DNase I resulted in a time-dependent release of receptor. A maximum of 60–70% of the [$^{125}$I]T3 was solubilized by the enzyme and approx. 30% of the receptor remained insensitive to nuclease digestion even after longer times of digestion or in the presence of higher concentrations of DNase I (not shown). Analysis of the kinetics of

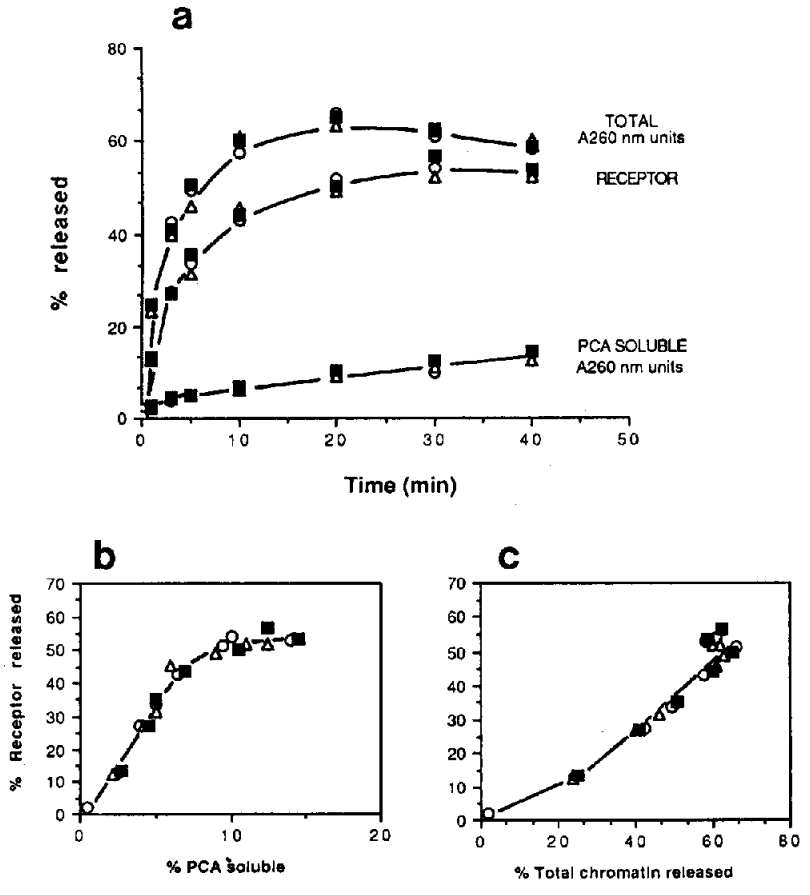


Fig.2. Micrococcal nuclease digestion of C6 cells nuclei. Nuclei from control (○), T3- (△) or butyrate (■)-treated cells were incubated with micrococcal nuclease and the extent of receptor and chromatin digestion determined. (a) Digestion kinetics, (b) receptor liberated vs PCA-soluble chromatin, (c) receptor release vs total chromatin solubilized.

digestion during the initial times of digestion demonstrated that the receptor was released 2–3-fold faster than the bulk of chromatin and 20-fold faster than the PCA-soluble fraction. After 3 min of incubation with the enzyme approx. 40% of the receptor was released in conditions where only 12% of total chromatin or 3% of PCA-soluble chromatin were solubilized. As can be observed in panels b and c of fig.1, a maximal amount of receptor was already digested when less than 30% of total chromatin or 10% of PCA-soluble chromatin was released. Fig.1 also shows that incubation with T3 (that decreased receptor levels by 37%) and with butyrate (that produced a 3-fold increase in receptor number) did not alter the kinetics of digestion of chromatin or the percent of receptor released.

The results obtained after limited digestion with MN are shown in fig.2. The preferential release of receptor over total chromatin was not observed after MN nuclease digestion and the amount of receptor increased almost linearly with the total chromatin released (fig.2c). The kinetics of receptor release were even somewhat slower than those found for the total chromatin fraction during the first 20 min (fig.2a). However, at initial times the receptor was solubilized 6-fold faster than the PCA-soluble chromatin and, as shown in fig.2b, a maximal amount of receptor was released when less than 10% of the chromatin was PCA-soluble. Additional receptor was not released with further increases of digestion (between 20 and 30% of PCA-soluble chromatin), a result very similar to that obtained with DNase I (fig.1b). It can be also observed in fig.2 that neither T3 nor butyrate altered the kinetics or extent of digestion by MN in C6 cells.

Fig.3 shows the sedimentation characteristics of the MN-solubilized receptors from control cells (panel A) and cells incubated with T3 (panel B) or butyrate (panel C). Analysis of the nuclear digests on sucrose gradients resulted in the separation of monomer (12.1 S), dimer (15.8 S), trimer (18.9 S) and larger nucleosome particles. The [$^{125}$I]T3 was predominantly found at approx. 6.5 S, where little if any DNA was found. Additionally, a less abundant 12.5 S receptor form, which sediments slightly more rapidly than the bulk of mononucleosomes generated was also observed in all groups. Incubation with T3 or butyrate altered the actual amount

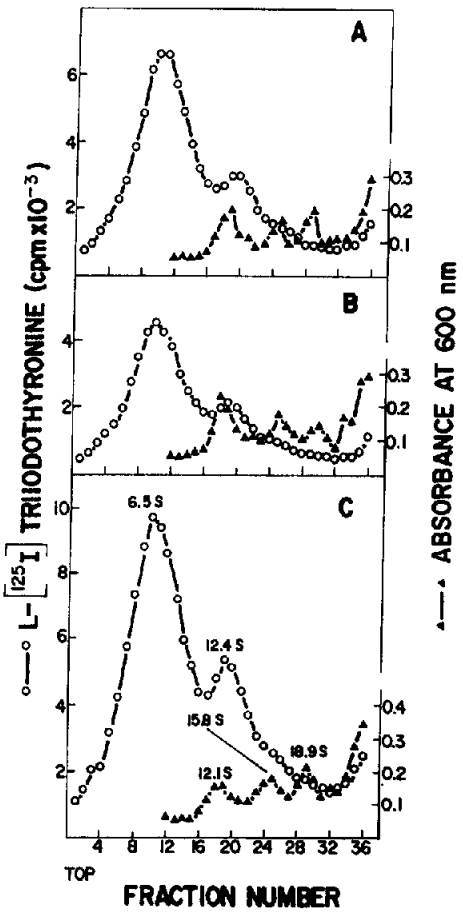


Fig.3. Sedimentation of T3 receptors released with micrococcal nuclease. Nuclei from control cells (A) and from cells incubated with T3 (B) or butyrate (C) were labeled with [$^{125}$I]T3, and digested with micrococcal nuclease. The solubilized material was centrifuged in isokinetic sucrose gradients and analyzed for [$^{125}$I]T3 (○) and for DNA by the method of Burton by measuring absorbance at 600 nm (▲).

of [$^{125}$I]T3 associated with both receptor forms. However, the pattern of sedimentation was similar in the different experimental groups and these treatments did not produce a change in the relative proportions of the 6.5 S and 12.5 S forms (70% and 20%, respectively).

To determine whether the nuclease-resistant receptors (30–40% of the total) were associated with the nuclear matrix, the nuclei were first digested and then extracted with KCl. As shown in table 1 digestion with DNase I released 63% of the specifically bound [$^{125}$I]T3 in control cells, and almost 70% of the radioactivity associated to the

Table 1

Determination of the nuclease and salt-resistant receptor fractions

| | Total [$^{125}$I]T3 (fmol) | DNase I pellet (fmol) | Super-natant (%) | 0.4 M KCl pellet (fmol) | Super-natant (%) | Nuclear matrix (%) |
|---|---|---|---|---|---|---|
| Control | 32 ± 1 | 12 ± 1 | 63 ± 5 | 4 ± 1 | 69 ± 2 | 11 ± 2 |
| T3 | 21 ± 1 | 7 ± 1 | 65 ± 4 | 2 ± 1 | 71 ± 2 | 9 ± 1 |
| Butyrate | 71 ± 4 | 22 ± 5 | 68 ± 6 | 8 ± 2 | 64 ± 1 | 11 ± 1 |

C6 cells nuclei were first digested with DNase I and then extracted with KCl. The percent of [$^{125}$I]T3-receptor released in each step as well as the percent in the residual pellet (a preparation of crude nuclear matrix) were calculated. Data are mean ± SD of triplicate cultures

undigested nuclear pellet was released after salt-extraction. The percentage of digested and extracted receptors did not vary after the treatment with T3 and butyrate, and in all cases the salt and nuclease-resistant [$^{125}$I]T3-receptor complexes that are presumably associated with the nuclear matrix represented only 9–11% of the total. Similar results were obtained when the extractions were carried out with higher salt concentrations (up to 2 M KCl), or when MN instead of DNase I was used (not shown).

## 4. DISCUSSION

We have examined the organization of the thyroid hormone receptor in the chromatin of C6 cells by digesting nuclei to varying extents with micrococcal nuclease or DNase I. The receptor is not randomly distributed in the chromatin but is rather preferently found in a fraction highly sensitive to nuclease digestion. This finding suggests that the T3 receptor is localized in 'active' chromatin, since transcriptionally competent chromatin is more susceptible to the action of nucleases [7]. Our results cannot distinguish between the possibility that the binding of receptor to chromatin induces a local alteration rendering the chromatin more sensitive to the enzyme, or that the receptors are preferentially bound to nuclease-sensitive sites. In this respect it is interesting to note that both thyroid hormone dependent and independent DNase I hypersensitive sites have been described in the vicinity of thyroid hormone-regulated genes [8,9].

The receptor appears to be associated with chromatin more sensitive to DNase I than to MN. The different accessibility of the receptor to digestion by both nucleases could be a consequence of the distribution of receptors between nucleosomes and linker regions since MN cleaves internucleosomal DNA, whereas DNase I shows no preference for linker DNA. As in other cell types [1,2] the receptor in C6 cells is predominantly associated with linker DNA (6.5 S receptor form) and in a smaller proportion with nucleosome particles (12.5 S form). The 6.5 S receptor contains a DNA fragment of 35–40 bases which is protected from digestion by the protein component [2]. Receptors activate transcription through high affinity binding to short 'hormone response elements' (HREs) in the target genes [10], but it is possible that much of the MN excised receptor represents receptors bound with low affinity to non-specific DNA sequences which may in fact obscure a smaller number of high affinity sites. It has been suggested that the protein component of the receptor released by MN could represent the native receptor interacting with other chromatin protein or another receptor molecule. The finding that the HREs present dyad symmetry suggests that the receptor binds as a dimer to DNA [10].

Recent studies suggest that a higher level of nuclear organization, the nuclear matrix, is associated with active gene transcription. In pituitary GC cells it has been reported that approx. 30–50% of the receptors were associated with nuclear matrix structures [11]. Our results suggest an important difference with respect to subnuclear localization of the receptor in C6 cells since, at least in our experimental conditions, only 10% of the receptor was salt- and nuclease-resistant.

Our study also documents for the first time the

effect of compounds that alter receptor levels on receptor distribution in chromatin. T3 and butyrate did not produce changes in receptor distribution between regions of chromatin with different accessibility to nucleases. The relative proportions of the 6.5 S and 12.5 S forms were not affected either, thus showing that the distribution of receptors between linkers and nucleosomes was not altered. These data suggest that changes in receptor number are not associated with redistribution between 'active' and 'inactive' regions of chromatin, although we cannot discard the possibility of specific changes in restricted domains of chromatin that cannot be detected with the methodology used.

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## REFERENCES

[1] Jump, D.B., Seelig, T., Schwartz, H.L. and Oppenheimer, J.H. (1981) Biochemistry 20, 6781–6789.
[2] Perlman, A.J., Stanley, F. and Samuels, H.H. (1982) J. Biol. Chem. 257, 930–938.
[3] Ortiz-Caro, J., Montiel, F., Yusta, B., Pascual, A. and Aranda, A. (1987) Mol. Cell. Endocrinol. 49, 255–263.
[4] Ortiz-Caro, J., Montiel, F., Pascual, A. and Aranda, A. (1986) J. Biol. Chem. 261, 13997–14004.
[5] Ortiz-Caro, J., Yusta, B., Montiel, F., Villa, A., Aranda, A. and Pascual, A. (1986) Endocrinology 119, 2163–2167.
[6] Burton, K. (1956) Biochem. J. 62, 315–323.
[7] Weintraub, H. and Groudine, M. (1976) Science 193, 848–856.
[8] Nyborg, J.K. and Spindler, S. (1986) J. Biol. Chem. 261, 5685–5688.
[9] Jump, D.B., Wong, N.C. and Oppenheimer, J.H. (1987) J. Biol. Chem. 262, 778–784.
[10] Beato, M. (1989) Cell 56, 335–344.
[11] Kumara-Siri, M.H., Shapiro, L.E. and Surks, M.I. (1986) J. Biol. Chem. 261, 2844–2852.