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Receptors from Glucocorticoid-Sensitive Lymphoma Cells and Two Classes of Insensitive Clones: Physical and DNA-Binding Properties

(tissue culture/altered receptors/sucrose gradients/DNA–cellulose)

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ABSTRACT **Mouse lymphoma tissue culture cells (S49.1A) are normally killed by dexamethasone, a synthetic glucocorticoid hormone. Dexamethasone-resistant clones have been selected from this line, some of which retain the ability to specifically bind dexamethasone. Addition of [^{3}H]dexamethasone to cultures, followed by cell fractionation, reveals that the nuclear transfer of hormone–receptor complexes in some of these variant clones is deficient (nt$^-$), while others show increased nuclear transfer (nt^i) relative to the parental line. Two independently selected members of each class have been studied here, in an effort to elucidate the molecular determinants involved in the receptor-nucleus interaction *in vivo*. The labeled receptors in cell-free extracts bind to DNA–cellulose, but only after previous incubation of the extract at 20°, similar to the treatment required for cell-free interaction of receptors with nuclei. More importantly, the apparent DNA-binding affinity of the nt$^-$ receptors is lower than the wild type, whereas the nt^i receptors bind DNA with an affinity higher than the parental molecules. The parallelism of nuclear and DNA binding, together with the observations that the receptors from the variants have sedimentation properties different from the wild-type cells, lead us to conclude that (*i*) these variants may contain altered receptor molecules and (*ii*) DNA is probably the primary nuclear binding site for steroid receptors *in vivo*.**

Steroid hormones appear to exert their primary action by a common mechanism (for review, see ref. 1) in which the hormone first becomes tightly bound by specific soluble receptor proteins in target cells. The hormone–receptor complexes then accumulate in the cell nuclei, where they are presumed to effect the transcription of specific genes. Thus, hormones might act as effector ligands that increase the affinity of the receptor for its nuclear site of action (2–4). Obviously, it is of great interest to characterize the nature of the nuclear binding sites. Many investigators have reported binding of radioactive steroid–receptor complexes to chromatin preparations (5–7), but to which specific component remains unclear. Thus, various studies suggest that receptors bind to nuclear membranes (8), to specific nuclear acidic (9) and basic proteins (10), and to purified DNA (3, 11–13). However, it is difficult to evaluate the significance of these phenomena *in vitro* because correlation with observations *in vivo* is either indirect or lacking altogether.

In an effort to overcome some of these difficulties, we have studied here the DNA-binding properties of glucocorticoid receptors from an established line of hormone-sensitive mouse lymphoma cells that are normally killed by the hormone (14, 15), and four independently selected hormone-resistant variant clones of that line. Data described in this report suggest that the four variants studied contain altered receptor proteins. Observations in whole cells show that two of the variant clones are greatly impaired in their ability to transfer receptor–steroid complexes to the nucleus, whereas the other two clones exhibit a striking increase in the percentage of receptor transferred. The present study shows that the extent of nuclear binding is closely paralleled by the cell-free binding of these receptors to purified DNA. These results strongly support the idea that DNA is a major determinant for nuclear localization in whole cells.

Abbreviations: nt$^-$ and nt^i, nuclear transfer deficient and increased nuclear transfer, respectively; dex, dexamethasone; wt, wild type.

MATERIALS AND METHODS

Cell Lines. The Balb/c mouse lymphoma line S49.1TB4.1A (here denoted S49.1A), cloned from a line established by Dr. A. Harris (16), was cultured in tightly stoppered spinner bottles in Dulbecco's modified Eagle's medium, supplemented with 10% horse serum and 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.4. Three of the variants described here, S49.1A.13R, S49.1A.22R, and S49.1A.55R, were originally selected in this laboratory by Dr. C. H. Sibley (14), and the fourth was obtained by the same method.

Preparation and Labeling of Cell Extracts. Cell pellets were homogenized with a motor-driven Teflon pestle in about one volume of TEGNO5 buffer, containing 10 mM Tris (pH 8.1), 1 mM Na_2EDTA, 10% (v/v) glycerol, 50 mM NaCl, 1 mM 2-mercaptoethanol, and 100 μg/ml of crystalline bovine serum albumin. The supernatant of a 15-min centrifugation at 33,000 × *g* was made 50 nM in the synthetic glucocorticoid [^{3}H]dexamethasone (27–35 Ci/mmol; New England Nuclear Corp.), and centrifuged for 75–90 min at 160,000 × *g*; the final supernatant was used as a source of [^{3}H]dexamethasone (dex)-labeled receptor protein.

DNA–Cellulose Chromatography. DNA–cellulose columns were prepared and run according to Alberts and Herrick (17), with Whatman CF-11 cellulose and calf thymus DNA (Worthington). Columns containing native or denatured DNA (0.7–1 mg per packed ml) gave equivalent results. Control experiments showed that little or no free [^{3}H]dex or [^{3}H]dex–receptor complex binds to blank cellulose under these conditions.

Other Methods are described in the legends to figures and tables.

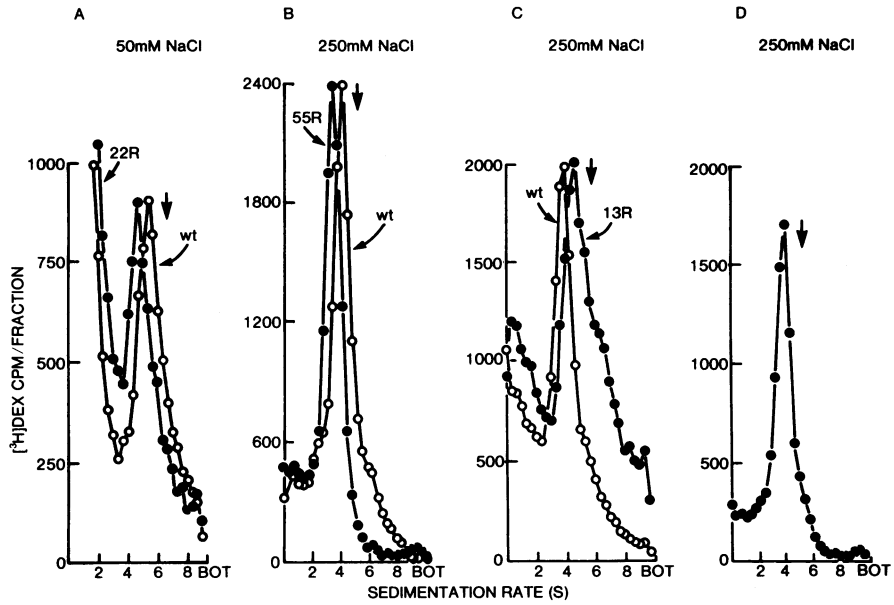

FIG. 1. Sucrose gradient sedimentation of [³H]dex-labeled extracts from wild-type (wt) and variant clones of S49.1A. Aliquots (100 μl) of extracts were sedimented through 5–20% sucrose gradients in buffer containing either 50 mM or 250 mM NaCl for about 18 hr at 234,000 × *g*. Sedimentation is plotted from left to right, and is calibrated in Svedberg units according to the enzyme activity peak of a 6.2S internal standard (*Escherichia coli* alkaline phosphatase) included in each gradient, and denoted by the vertical arrow. In some cases (B and C), the extracts were first treated with small amounts of activated charcoal [10% of the amount normally used to quantitate labeled receptor (18)] to remove most of the [³H]dex. Controls showed that this treatment does not effect sedimentation properties of the receptors. (A) 50 mM NaCl; O, wt; ●, 22R. (B) 250 mM NaCl; O, wt; ●, 55R. (C) 250 mM NaCl; O, wt; ●, 13R. (D) 250 mM NaCl eluate of wt receptor from DNA–cellulose.

RESULTS

In Vivo Distribution of Bound Dex. In order to compare the nuclear localization of receptors in whole cells with cell-free DNA-binding, we chose to study variant clones whose receptors have functional hormone-binding sites but are aberrant in their nuclear binding properties. Thus, cells were incubated in growth medium in the presence of [³H]dex for a sufficient time to label the receptors and allow their intracellular redistribution. Specifically bound [³H]dex (see legend to Table 1) was then determined in the crude nuclear and cytoplasmic fractions of cell homogenates (14). As shown in Table 1, the "wild-type" parental line, S49.1A, transfers about half of it labeled receptors to the nucleus. In contrast, two of the variant lines, S49.1A.13R and S49.1A.22R, display greatly reduced nuclear binding activity, whereas two other clones, S49.1A.55R and S49.1A.75R, show significantly more nuclear binding than S49.1A (Table 1). The "13R" and "22R" lines are phenotypically classified as nt^-, or nuclear transfer deficient, and the "55R" and "75R" lines as nt^i, or increased nuclear transfer.

TABLE 1. *Intracellular distribution of bound [³H]dex after labeling in vivo*

Clone	% Specifically bound [³H]dex in nuclear pellet	Phenotypic classification
S49.1A	50 (48–54)	wt, wild type
13R	26 (22–31)	nt^-, nuclear transfer deficient
22R	8 (0–16)	
55R	85 (77–94)	nt^i, increased nuclear transfer
75R	93 (87–100)	

Aliquots of cells (2.5 to 7.5 × 10^7/0.5 ml) were incubated in medium containing 20 nM [³H]dex. A parallel culture contained, in addition, a 500- to 1000-fold excess of unlabeled dex. The crude nuclear and cytoplasmic fractions were prepared as described (14); specifically bound [³H]dex in each fraction, defined as the difference in [³H]dex cpm bound in the presence and absence of unlabeled dex, was determined. Values given are the mean and range of 2–4 determinations.

Sucrose Gradient Sedimentation of [³H]dex-labeled extracts from the wild-type and variant lines was done in buffers

TABLE 2. *Sedimentation properties of [³H]dex-labeled receptors*

Clone	Sedimentation rate (S) 50mM NaCl	250 mM NaCl
S49.1A	5.5 (5.2–5.9)	4.0 (3.9–4.2)
13R	6.4 (6.2–6.7)	4.4 (4.3–4.7)
22R	4.6 (4.5–5.7)	4.0 (3.9–4.0)
55R	5.5 (5.4–5.6)	3.5 (3.4–3.5)
75R	5.4 (5.3–5.6)	3.5 (3.4–3.5)

Sucrose gradients [5.1 ml; 5–20% (w/v) sucrose, Mann Special enzyme grade] were prepared according to Martin and Ames (21), and contained the same buffer as the sample to be applied. To each 100-μl sample was added about 10 μg of *E. coli* alkaline phosphatase as a 6.2S internal standard. Gradients were sedimented at 2° for 17–24 hr at 234,000 × *g*. Fractions were assayed for alkaline phosphatase activity as described (22). Values for the S49.1A receptor are from 15 determinations; others are from 2–5 determinations.

containing 50 mM and 250 mM NaCl. In order to detect small differences in the sedimentation rate, the gradients were formed and collected especially carefully, as indicated by the fact that the peak of the marker alkaline phosphatase activity (6.2 S) was found in the same fraction of each gradient in a given centrifugation. As shown in Fig. 1, the receptor in wild-type extracts sediments as a relatively homogeneous 4S peak at the high salt concentration and as a somewhat broader peak, centered at about 5.5 S, in low salt. This behavior is very similar to that of other steroid receptors (18–20), which also decrease in sedimentation rate to about 4 S as the ionic strength is increased. Other experiments reveal that the 4S and 5.5S receptor forms are freely interconverted by adjusting the ionic strength of the buffer. Furthermore, these experiments suggest that the 5.5S form may result from association of nonreceptor components with the 4S receptor (K. Yamamoto, unpublished results). In any case, we were surprised to find small, but reproducible, differences between the sedimentation properties of the wild type and each of the variant receptors studied. As shown in Table 2, the 13R receptor sediments more rapidly than the wild-type molecule at both low and high salt concentrations, whereas the 22R receptor migrates more slowly at low salt concentration, but is indistinguishable from wild type at high salt concentration. The nt^i (55R and 75R) receptors behave alike, sedimenting "normally" at low salt concentration, but more slowly than wild-type receptors at high salt concentration. Fig. 1A-C illustrates examples of typical sedimentation patterns obtained, and Table 2 summarizes the results of several such experiments.

Receptors Binding to DNA Requires "Activation." To assess the binding of the various receptors to DNA, cell extracts were prepared in buffer containing 50 mM NaCl and chromatographed on DNA–cellulose columns. Only a minor proportion of the labeled receptor put onto the column (an average of 8% in wild type) binds to the DNA if the extract is kept cold throughout the procedure (Table 3). However, if the extract is incubated at 20° for 35 min before chromatography at 4°, the DNA-binding of receptor–steroid complex increases by about an order of magnitude. This phenomenon has been termed "activation," and has been shown to stimulate cell-free receptor binding to intact nuclei and DNA (23–25). Although the physical changes occurring upon activation are not understood, preliminary experiments show that the 20° incubation does not result in a detectable change in the sedimentation properties of the receptors (K. Yamamoto, unpublished), unlike the 4S to 5S conversion observed with estradiol receptors under similar conditions (3, 22).

DNA-Binding and Elution of Variant Receptors. Table 3 shows that both nt^- receptors bind poorly to DNA–cellulose even after activation at 20°. However, these data also show that none of the variants appears to be defective in activation, since in every case, the 20° incubation strongly stimulates binding. Under conditions where more than 85% of the input wild-type receptor binds DNA, only 10–15% of the nt^- receptors bind. This small fraction bound appears to be an equilibrium figure, since it does not increase as the duration of contact between the extract and the DNA–cellulose is lengthened from 20 min to 4 hr. Furthermore, both bound and unbound fractions were reloaded onto DNA–cellulose. About the same low percentage binding occurred with each, showing that the binding observed is not due merely to heterogeneity in the receptor population (data not shown). Moreover, the variant receptors elute from DNA–cellulose at 110–120 mM NaCl, clearly lower than the 170 mM NaCl required to elute wild-type receptors (Table 3 and Fig. 2A).

The nt^i receptors, which show abnormally *high* nuclear localization in whole cells, bind like wild type, almost quantitatively to DNA–cellulose columns after 20° activation (Table 3). However, these variant receptors require 210 mM NaCl to be eluted from the DNA, a significantly higher concentration than that needed for elution of wild-type receptor (Table 3 and Fig. 2B).

Although the order of elution in a gradient of increasing ionic strength is not a rigorous measure of the relative affinities of these receptors for DNA, these results strongly support the notion that the receptors from the nt^- variants bind to DNA with affinities lower than wild type, whereas those from the nt^i cells bind with higher affinity than the parental line. Moreover, recent experiments using partially purified receptors from wild type and an nt^- extract (22R) indicate that at 50 mM NaCl the equilibrium dissociation constant (4) for the parental receptor–DNA interaction is nearly two orders of magnitude lower than that for the variant receptor (K. Yamamoto, unpublished).

DNA-Binding in Mixed Extracts. An alternative explanation for the DNA-binding properties of the receptors from variant lines is that inhibitory substances, present in the normal cell extracts, are more active in nt^- and less active in nt^i extracts. Still another possibility is that components that stimulate receptor binding might act in the nt^i and wild-type extracts, but not in the nt^- extracts. Two types of cell-free complementation experiments were carried out to determine if such activities could be detected.

In one experiment, wild-type and nt^- extracts were prepared and the receptors were bound with either ^{3}H-labeled or unlabeled dex. They were mixed by pairs in the combinations shown in Table 4, incubated at 20°, and chromato-

TABLE 3. *Binding of receptors to DNA*

Clone	% Input receptor bound after 0–4° incub.	% Input receptor bound after 20° incub.	mM NaCl to elute bound receptor
S49.1A	8 (5–12)	86 (75–100)	170
13R	3 (2–3)	15 (12–23)	120
22R	1 (0.6–2)	9 (6–14)	110
55R	8 (5–11)	82 (74–90)	210
75R	12 (8–16)	91 (82–100)	210

Input [^{3}H]dex–receptor complex was quantitated by subjecting an aliquot of extract either to treatment with activated charcoal to remove free [^{3}H]dex (2) or, in some cases, to sucrose gradient sedimentation. Extracts containing 10,000–50,000 cpm of [^{3}H]-dex-labeled receptor in 0.5 ml of TEGNO5 buffer were put onto 1-ml DNA–cellulose columns, pumped at a constant rate of 3–4 column volumes/hr. After the unbound material was rinsed out with 6 column volumes of TEGNO5, bound receptor was eluted either stepwise, with buffer containing 250 mM NaCl, or with a 12 column volume linear gradient from 50 mM to 300 mM NaCl. Fractions (200–300 μl) were collected directly into counting vials. NaCl concentrations were determined on 5-μl aliquots, with a Radiometer conductivity meter. Values given are the mean of at least 2 determinations.

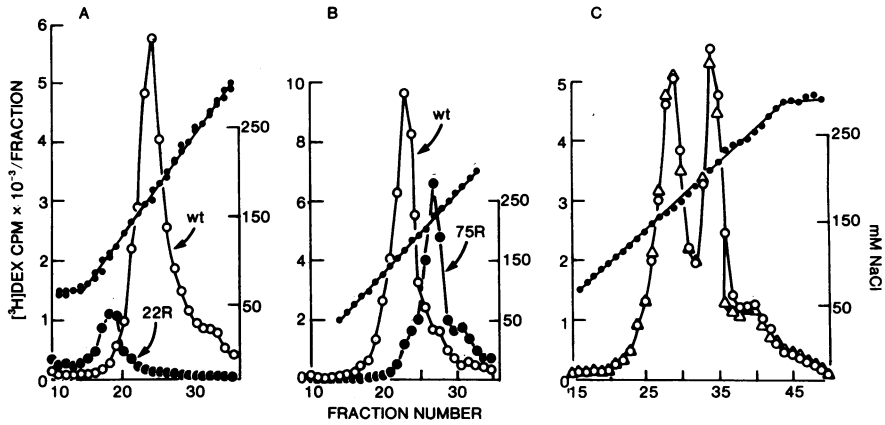

FIG. 2. Elution of receptors bound to DNA–cellulose with 50–300 mM NaCl gradients. In each case, extracts in TEGNO5 were loaded by pairs onto separate DNA–cellulose columns. After the columns were rinsed free of unbound material, bound receptors in the two columns were eluted with a NaCl gradient drawn from a single mixing chamber. Fractions (about 250 μl) were collected directly into counting vials; the NaCl gradient was measured on a 5-μl aliquot of each fraction. •, NaCl gradient. (A) O, wt; ●, 22R. Identical results are obtained with 13R. (B) O, wt; ●, 75R. Identical results are obtained with 55R. (C) O, wt +75R, incubated at 20° before they were mixed, △, wt +75R, incubated at 20° after they were mixed. Identical results are obtained with wt +55R.

graphed on DNA–cellulose. The results, shown in Table 4 for 22R, demonstrate that the extracts behave independently, with no inhibition of wild-type binding by nt⁻, and no stimulation of nt⁻ binding by wild type.

The second type of experiment was carried out with wild-type and nt^i extracts, *both* of which were labeled with [³H]dex. Aliquots of the extracts were mixed, either before or after the 20° incubation, then loaded onto DNA–cellulose columns; the bound components were gradient-eluted. The results, depicted in Fig. 2C for 7SR, again show that substances present in one extract do not affect the DNA-binding properties of the other, whether the two extracts are mixed before or after the 20° incubation.

These data imply that the aberrant DNA-binding properties of the receptors from the variants studied here are due to alterations in the receptor molecules themselves, rather than to alterations in other soluble components in the extracts.

DISCUSSION

Ten different nt⁻ and nt^i clones have been phenotypically characterized in this laboratory. In addition, more than 100 clones that lack hormone-binding activity (r⁻, or "receptor activity deficient") have been selected. Like other variant-phenotypes in cell culture systems (26–28), hormone resistance in S49.1A arises at a relatively high frequency (about 3×10^{-6}) in pseudodiploid as well as pseudotetraploid clones (14). Although it is encouraging that mutagenesis and fluctuation analysis studies (14) are both consistent with the possibility that these variants represent actual structural gene mutations, direct demonstration of a molecular alteration in a purified component would be of obvious value. It is interesting that all of the variant lines that have been studied show defects in the receptor system, rather than in a nuclear locus with an "acceptor" function.

TABLE 4. *DNA binding in wt × nt⁻ mixed extracts*

[³H]Dex extract	Unlabeled dex extract	% [³H]dex receptor bound
S49.1A	S49.1A	75
S49.1A	22R	81
22R	S49.1A	3.6
22R	22R	2.2

Extracts of S49.1A and 22R were prepared in buffer containing 80 nM NaCl (to maximize the differential binding of the two receptors), divided, and treated with radioactive and nonradioactive dex. Aliquots of extracts containing 15,000 cpm of [³H]-dex-labeled receptor were mixed with an equal volume of unlabeled extract as indicated, incubated 35 min at 20°, and chromatographed on DNA–cellulose as described in the legend to Table 3.

Gehring and Tomkins (25) have recently characterized an nt⁻ variant in detail, and conclude that glucocorticoid receptors contain separate binding "domains": one for steroid and the other for the nuclear binding sites. Our results support the possibility that the four variants described in this report produce receptors with altered nuclear binding domains, differing from the wild-type molecule in their affinity for DNA, as well as their behavior in sucrose gradients.

Previous experiments with various steroid receptors have provided correlative evidence that DNA binding *in vitro* may be directly related to nuclear localization *in vivo*. First, the receptors must be complexed with hormone in order to bind either nuclei or DNA (3, 11). Second, a time- or temperature-dependent activation process required for nuclear transfer is also required for DNA-binding (3, 11). Third, both nuclear and DNA-binding of the estradiol receptor result in conversion of the 4S receptor to a 5S form (3, 22, 29). Fourth, the "activation" or "conversion" of glucocorticoid and estradiol receptors gives increased affinities for DNA, but not for RNA (ref. 4; Rousseau *et al.*, in preparation). Finally, supoptimal- or anti-inducer compounds, which bind to receptors but do not promote their nuclear localization, also do not stimulate the receptor–DNA interaction (3, 11).

In this study, the question of the biological relevance of receptor–DNA binding has been approached in a different way. Here, we have exploited the observation that the receptors in certain hormone-insensitive clones of the mouse lymphoma cell line S49.1A display either increased or decreased amounts of nuclear binding. Using these variants, we have been able to directly correlate differences in nuclear

binding in intact cells with changes in the apparent affinity of the receptors for purified DNA in cell-free extracts. Of course, it is conceivable that the variant receptors are also modified in their affinities for nuclear components other than DNA. Although this possibility has not been ruled out, the altered DNA-binding properties reported here would, by themselves, change the whole cell nuclear localization characteristics in the ways observed, since sites are certainly available for protein–DNA interaction in the intact nucleus. Thus, in the simplest interpretation, these results strongly support the contention that DNA is the primary nuclear binding site for glucocorticoid receptors in these mouse lymphoma cells *in vivo*.

Using the methods described here, we have not been able to approach the question of whether the receptor can recognize a specific DNA base sequence and bind to it with high affinity. In general, DNA–cellulose chromatography is not suited to the detection of specific site binding, due to the high relative concentrations of nonspecific DNA sequences. Thus, even proteins with very high affinities for specific sequences bind readily to nonspecific DNA under these conditions (30).

Nevertheless, it is of interest that the requirement for activation, and the hierarchy of whole cell nuclear binding observed for wild-type and variant receptors is faithfully preserved in cell-free binding to nonhomologous DNA. One interpretation of this result is that the binding observed in whole cells may itself be to nonspecific sequences. Indeed, the nuclear binding of estrogen *in vivo* (31) and glucocorticoid receptors (J. Baxter and S. Simons, personal communication) is of low affinity and to a large number of sites.

At present, we cannot exclude the possibility that steroid action requires only interaction of the receptors with any DNA sequence. Alternatively, it has recently been proposed (ref. 4; Yamamoto and Alberts, submitted) that steroid receptors might function by binding to only a few high affinity chromosomal sites whose specificity is masked by the vast excess of low affinity binding. The existence of the nt^i variant class implies that the first model, in its most extreme case, is incorrect. Moreover, the apparently paradoxical binding behavior of the nt^i receptors can be easily explained in terms of the second model. That is, the 55R and 75R receptors may have an increased affinity for nonfunctional, relative to biologically functional, genome binding sites, resulting in increased nuclear binding together with loss of the hormone response.

In any case, we have concluded that nuclear localization of steroid receptors *in vivo* is probably the result of binding to DNA. Whether this low affinity binding of a large number of receptors is the direct determinant of the biological response, or if instead a small number of high affinity sites also exist, remains an unresolved issue.

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