Boffa, L. C., Gruss, R. J., & Alfrey, V. G. (1981) *J. Biol. Chem. 256*, 9612–9621.
Bradbury, E. M., Inglis, R. J., Matthews, H. R., & Langan, T. A. (1974) *Nature (London) 249*, 553–556.
D'Anna, J. A., Tobey, R. A., & Gurley, L. R. (1980) *Biochemistry 19*, 2656–2671.
D'Anna, J. A., Gurley, L. R., & Becker, R. R. (1981) *Biochemistry 20*, 4501–4505.
Delegeane, A., & Lee, A. (1982) *Science (Washington, D.C.) 215*, 79–81.
Glover, C. V., Vaura, K. J., Guttman, S. D., & Gorovsky, M. A. (1981) *Cell (Cambridge, Mass.) 23*, 73–77.
Gurley, L. R., Walters, R. A., & Tobey, R. A. (1974) *J. Cell Biol. 60*, 356–364.
Gurley, L. R., Walters, R. A., & Tobey, R. A. (1975) *J. Biol. Chem. 250*, 3936–3944.
Hohmann, P. (1983) *Mol. Cell. Biochem. 57*, 81–92.
Inglis, R. J., Langan, T. A., Matthews, H. R., Hardie, D. G., & Bradbury, E. M. (1976) *Exp. Cell Res. 97*, 418–425.
Johnson, E. M., & Allfrey, V. G. (1978) *Biochem. Actions Horm. 5*, 1–11.
Kleinsmith, L. J., Allfrey, V. G., & Mirsky, A. E. (1966) *Proc. Natl. Acad. Sci. U.S.A. 55*, 1182–1189.
Kruh, J. (1982) *Mol. Cell. Biochem. 42*, 65–82.
Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.
Langan, T. (1968) *Science (Washington, D.C.) 162*, 579–580.
Langan, T. A. (1971) *Ann. N.Y. Acad. Sci. 185*, 166–180.
Langan, T. A. (1982) *J. Biol. Chem. 257*, 14835–14846.
Louie, A. J., & Dixon, G. H. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1975.
Ord, M. G., & Stocken, L. A. (1966) *Biochem. J. 98*, 888–897.
Panyim, S., & Chalkley, R. (1969) *Biochem. Biophys. Res. Commun. 37*, 1042–1049.
Pehrson, J. R., & Cole, R. D. (1982) *Biochemistry 21*, 456–460.
Prosad, K., & Sinha, C. (1976) *In Vitro 12*, 125–132.
Ruiz-Carrillo, A., Wangh, L. J., & Allfrey, V. (1975) *Science (Washington, D.C.) 190*, 117.
Sung, M. T., Harford, J., Bundman, M., & Vidalomas, G. (1977) *Biochemistry 16*, 279–285.
Wagner, T. E., Harford, J. B., Serra, M., Vandegrift, U., & Sung, M. T. (1977) *Biochemistry 16*, 286–290.

Chromatin Structure of the Chicken Lysozyme Gene Domain As Determined by Chromatin Fractionation and Micrococcal Nuclease Digestion†

Wolf H. Strätling* and Albert Dölle
Physiologisch-Chemisches Institut, D-2000 Hamburg 20, FRG
Albrecht E. Sippel
Zentrum für Molekulare Biologie der Universität Heidelberg (ZMBH), D-6900 Heidelberg, FRG
Received June 3, 1985

ABSTRACT: The chromatin structure encompassing the lysozyme gene domain in hen oviduct nuclei was studied by measuring the partitioning of coding and flanking sequences during chromatin fractionation and by analyzing the nucleosome repeat in response to micrococcal nuclease digestion. Following micrococcal nuclease digestion, nuclei were sedimented to obtain a chromatin fraction released during digestion (S1) and then lysed in tris(hydroxymethyl)aminomethane–(ethylenedinitrilo)tetraacetic acid–[ethylenebis(oxyethylenenitrilo)]tetraacetic acid and centrifuged again to yield a second solubilized chromatin fraction (S2) and a pelleted fraction (P2). By dot-blot hybridization with 14 specific probes, it is found that the fractionation procedure defines three classes of sequences within the lysozyme gene domain. The coding sequences, which partition with fraction P2, are flanked by class I flanking sequences, which partition with fractions S1 and P2 and which extend over 11 kilobases (kb) on the 5′ side and probably over about 4 kb on the 3′ side. The partitioning of class II flanking sequences, which are located distal of class I flanking sequences, is different from that of class I flanking sequences. Coding sequences lack a canonical nucleosome repeat, class I flanking sequences possess a disturbed nucleosome repeat, and class II flanking sequences generate an extended nucleosomal ladder. Coding and class I flanking sequences are more readily digested by micrococcal nuclease than class II flanking sequences and the inactive β^A-globin gene. In hen liver, where the lysozyme gene is inactive, coding and class I flanking sequences fractionate into fractions S2 and P2. Chromatin fractionation of steroid-induced and deinduced chick oviduct nuclei shows a close correlation between active transcription of the lysozyme gene and enrichment of coding sequences in fraction P2. Our results indicate that the partitioning of coding lysozyme gene sequences with low-salt insoluble nuclear material relates to the transcriptional process along these sequences.

The packaging of transcribed genes into a less condensed chromatin structure has been visualized by electron microscopy (Beermann, 1952; Foe et al., 1976) and has been investigated by use of nuclease digestion (Weintraub & Groudine, 1976). Active genes and genes that have been transcribed exhibit an elevated sensitivity to digestion with various nucleases. A more detailed analysis performed on many genes revealed that often a distinct length of flanking sequences is equally as sensitive to nuclease digestion as the coding sequences. In addition, active genes exhibit sites that are at least 1 order of magnitude more sensitive to nucleases than the remainder (Elgin, 1981).

† This work was supported by grants from the Deutsche Forschungsgemeinschaft (Str 145/9) to W.H.S. and the Bundesministerium für Forschung und Technologie (BCT 0364/1) to A.E.S.
* Address correspondence to this author.

0006-2960/86/0425-0495$01.50/0

These sites are located in the flanking regions, and one of them usually is close to the start site of transcription. A new aspect of the structure of active chromatin was suggested by the observation that active sequences are enriched in an insoluble fraction (matrix) obtained from nuclei by treatment with 2 M NaCl and nuclease digestion (Jackson et al., 1981, 1982; Robinson et al., 1982, 1983; Ciejek et al., 1983; Hentzen et al., 1984; Jost & Seldran, 1984).

Chromatin fractionation has attracted the efforts of many investigators, probably because a reliable method would enable a characterization of the structural and compositional features of active and inactive chromatin. A salt fractionation procedure, first introduced by Sanders (1978), solubilizes at low salt concentrations specifically sequences that have been transcribed in mature hen erythrocyte nuclei (Rocha et al., 1984). A fractionation technique using separation of the chromatin released from nuclei during nuclease digestion followed by extraction with EDTA[1] has been developed by Bloom & Anderson (1978). They showed that the ovalbumin gene from hen oviduct chromatin was enriched in the fraction released during digestion, as assayed by liquid hybridization with a cDNA probe. Rose & Garrard (1984) used a very similar technique and found that the transcribed κ immunoglobulin light chain genes are associated with insoluble nuclear material, as assayed by dot-blot hybridization. These conflicting reports prompted us to employ a gentle low-salt fractionation procedure to perform a detailed analysis of the partitioning of 14 sequences of the lysozyme gene domain in hen oviduct chromatin. We observed a differential partitioning of transcribed coding, nontranscribed coding, flanking, and inactive sequences. Transcribed coding sequences are characterized by the lack of a canonical nucleosome repeat and by a specific partitioning with low-salt insoluble nuclear material.

Materials and Methods

Isolation and Digestion of Nuclei and Fractionation of Chromatin. Nuclei were freshly prepared from the oviduct of laying hens (Lohmann Selected Leghorn). The magnum portion was homogenized in a nuclear buffer (40 mM NaCl, 60 mM KCl, 10 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 5 mM butyrate, 0.14 mM spermidine) containing 0.5 M sucrose, 0.5 mM phenylmethanesulfonyl fluoride, 0.5% Triton X-100, and 0.5% sodium deoxycholate by three 30-s bursts of a Janke & Kunkel Ultraturrax. After sequential passage through one and four layers of cheesecloth, nuclei were pelleted at 4000 rpm in the HB4 rotor of a Sorvall centrifuge. The pelleted nuclei were resuspended in nuclear buffer containing 1.8 M sucrose and resedimented at 8000 rpm. The purified nuclei were suspended in nuclear buffer at an optical density A_{260} = ~50, and following addition of $CaCl_2$ to 0.5 mM, they were digested with 20 units of micrococcal nuclease (Boehringer) per milliliter of nuclear suspension at 25 °C for 30 min. This extent of digestion degrades approximately 1.9% of nuclear DNA into perchloric acid soluble material. After digestion, the suspension was quickly chilled and centrifuged at 5000 rpm in the HB4 rotor. The supernatant (S1) was saved, and the remaining pellet was suspended in 10 mM Tris-HCl, pH 7.5, 1 mM Na-EDTA, and 1 mM Na-EGTA and incubated for 45 min at 0 °C. Then, the samples were centrifuged at 10000 rpm to give a second supernatant (S2) and a second pellet (P2). Hen liver nuclei were prepared, digested, and fractionated in the same manner as oviduct nuclei.

To extract the DNA, the samples were made 10 mM, 1 mM, and 0.2% with respect to Na-EDTA, Na-EGTA, and Sarcosyl, respectively, and digested successively with RNase A at 37 °C for 1 h and with proteinase K at 37 °C overnight, each at 50 μg/mL. DNA purification was then continued by extraction with phenol–chloroform–isoamyl alcohol as previously described (Britten et al., 1974). Agarose (1%) slab gels were run as previously described (Strätling & Klingholz, 1981).

Hybridization. To measure the abundance of specific sequences by dot-blot hybridization, the DNA samples were diluted to about 100 μg/mL. Aliquots containing multiples of 1, 2, and 4 μg of DNA were made 0.4 N with respect to NaOH. Following incubation for 15 min at 20–25 °C, the samples were chilled and an equal volume of 2 M ammonium acetate was added. Aliquots containing 1, 2, and 4 μg of DNA were loaded onto a nitrocellulose filter, which was pretreated with Kafatos solution (1 M ammonium acetate–0.02 M NaOH) (Kafatos et al., 1979), by using a hybridot apparatus (Bethesda Research Laboratories). Controls have shown that fragments as small as 145 bp also bound quantitatively to the filter. The filter was baked for 2 h in a vacuum oven at 80 °C, prehybridized overnight at 65 °C with 4 × SSC (1 × SSC = 150 mM NaCl, 15 mM sodium citrate), 5 × Denhardt's reagent (Denhardt, 1966), and 100 μg/mL denatured, sonicated salmon sperm DNA, and then hybridized overnight at 65 °C in 4 × SSC, 10 mM Na-EDTA, 0.1% SDS, 5 × Denhardt's reagent, 20 μg/mL salmon sperm DNA, and 1.0 ng of a specific ^{32}P-labeled nick-translated plasmid probe (specific activity, approximately 7×10^7 dpm/μg of DNA) (Rigby et al., 1977) per cm^2 of the filter. The volume of the hybridization reaction was 30% in excess of the volume to wet the filter. The filter was then washed at 65 °C sequentially with 2 × SSC, 1 × SSC, and 0.2 × SSC (each in 25 mM sodium phosphate buffer, pH 6.5, 0.1% SDS) twice with each solution for 30 min. Fuji RX film was exposed to the filter for several days at −80 °C. The intensity of each dot on the film was determined by scanning at 546 nm in an Eppendorf photometer.

Southern blot hybridization was performed according to Southern (1975), but with Kafatos solution for blotting and the hybridization protocol described above.

Chicks. Primary stimulation of immature 6-week-old chicks was performed by subcutaneous implantation of silicone tubes filled with diethylstilbestrol (Hynes et al., 1979). Following 12 days of induction, the tubes were removed and the chicks were kept without receiving any hormone for 5 days (withdrawn chicks). Following hormone withdrawal the chicks received daily injections of 2.5 mg of diethylstilbestrol dissolved in sesame oil for 6 days (restimulated chicks).

Probes. All DNA probes result from subcloned DNA fragments of λLys-30, λLys-31 (Lindenmaier et al., 1979; Nowock & Sippel, 1982), and pFF$_2$-Lys-16 (Baldacci et al., 1981). The chicken adult β-globin gene fragment is a subclone of pβ1EB15 derived from the λ clone λCβG1 (Dolan et al., 1981, 1983).

Results

We have investigated the partitioning of coding and noncoding sequences of the chicken lysozyme gene domain by a low-salt chromatin fractionation procedure in order to study the chromatin structure encompassing this gene. Nuclei were isolated from the oviduct of laying hens and digested with

[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, (ethylenedinitrilo)tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; kb, kilobase(s); kbp, kilobase pairs; SSC, standard saline citrate.

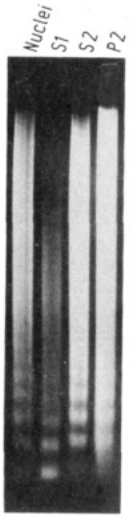

FIGURE 1: Fractionation of hen oviduct chromatin by a low-salt procedure. Nuclei from hen oviduct were digested with micrococcal nuclease, and the chromatin was separated into fractions S1, S2, and P2 by a low-salt fractionation procedure. The purified DNA from these fractions was electrophoresed on a 1% agarose gel followed by staining with ethidium bromide.

micrococcal nuclease under conditions that preserve nuclear morphology (see Materials and Methods). A first low-speed centrifugation step separated the nuclei from a chromatin fraction (S1) released during nuclease digestion. This chromatin fraction contains about 10% of the nuclear DNA (Table I) and consists mostly of mononucleosomes and short-chain oligonucleosomes as shown by agarose gel electrophoresis of the purified DNA (Figure 1). After treatment of the pelleted nuclei with Tris–EDTA–EGTA, another low-speed centrifugation step yielded a second soluble chromatin fraction (S2) and a pelleted fraction (P2) containing about 60% and 30%, respectively, of the nuclear DNA (Table I). The relative abundance of 14 unique DNA sequences of the lysozyme gene domain in each chromatin fraction was then determined by dot-blot hybridization with specific ^{32}P-labeled nick-translated probes (Figure 2 and Table I). Together, these probes span over 16.8 kb and derive from a chromosomal region 29.9 kb long. As shown in Figure 2B, the probes used are complementary to the coding region of the gene as well as to non-transcribed upstream and downstream flanking sequences. Relative to the hybridization signals exhibited by total DNA prepared from unfractionated nuclear digests, the transcribed sequences g, h, and i are 3.2- to 4.1-fold enriched in fraction P2—very close to the maximum possible enrichment for a sequence representing 3.4-fold. In fractions S1 and S2, the transcribed sequences are significantly depleted, showing that these fractions contain very little (≈10%) of these sequences. Therefore, the great majority of transcribed sequences are present in the low-salt insoluble chromatin fraction P2. This partitioning is faithful in that, when fraction P2 is challenged again by extraction with Tris–EDTA–EGTA, very little sol-

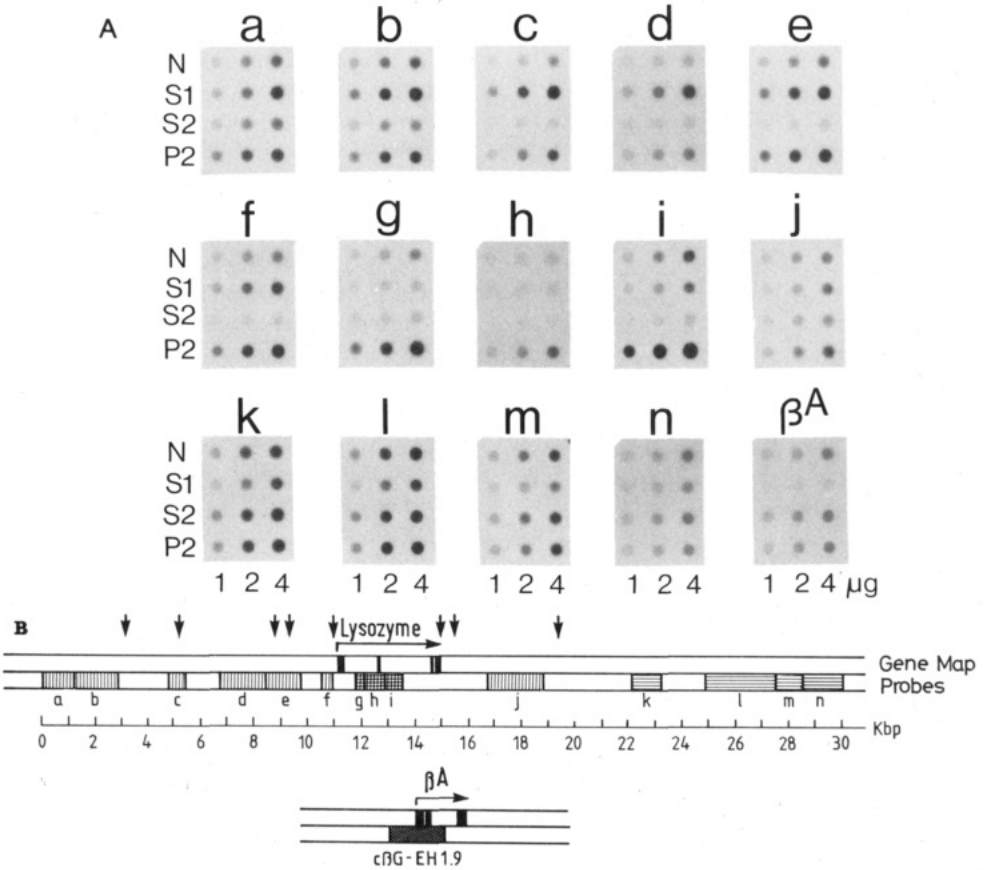

FIGURE 2: Three classes of sequences within the lysozyme gene domain as defined by differential partitioning during chromatin fractionation. (A) Hen oviduct nuclei were digested with micrococcal nuclease, and chromatin fractions S1, S2, and P2 were prepared. Purified DNA from these fractions was dot-blotted at the indicated amounts onto nitrocellulose filters. The filters were hybridized with the indicated ^{32}P-labeled probes and subjected to autoradiography. (B) Location of the hybridization probes relative to the genomic organization. The uppermost lanes show the map of the lysozyme gene and of the adult β-globin (β^A) gene with the exons represented by black bars. The lane below the lysozyme gene map shows the location of the hybridization probes and indicates the sequence category defined by partitioning during chromatin fractionation: crosshatched bars, coding sequences; vertical bars, class I flanking sequences; horizontal bars, class II flanking sequences. The horizontal arrows indicate the direction of transcription, and the vertical arrows above the lysozyme gene map refer to previously mapped hypersensitive sites (Fritton et al., 1983). The scale of the β^A gene is the same as that of the lysozyme gene map.

Table I: Partitioning of Lysozyme Gene Coding and Flanking Sequences during Hen Oviduct Chromatin Fractionation

chromatin fraction	% of nuclear DNA[a]	x-fold enrichment of fragment[b] for sequence category[c]														
		class I flanking						coding			cI-f	class II flanking				inact
		a	b	c	d	e	f	g	h	i	j	k	l	m	n	β^A
S1	10.5	1.16	1.68	4.2	2.52	1.96	1.74	0.30	0.37	0.56	0.95	0.87	0.73	0.79	0.72	0.14
S2	59.8	0.87	0.62	0.26	0.43	0.24	0.23	0.27	0.16	0.19	0.87	1.18	1.08	1.16	1.17	1.26
P2	29.7	1.55	1.48	1.66	1.51	2.14	2.66	3.53	3.21	4.15	1.17	1.13	1.12	1.16	1.07	1.03

[a] Mean of eight experiments. [b] The factors of enrichment were calculated from the relative intensities of the dots in the autoradiogram in Figure 2A as measured by scanning at 546 nm. The values obtained were confirmed after autoradiography by cutting out each dot and determining the radioactivity retained on the filter by liquid scintillation. [c] The sequence category refers to the differential distribution of the respective sequences during chromatin fractionation and to the appearance of a nucleosome repeat pattern in response to micrococcal nuclease digestion (see text): cI-f, class I flanking sequence; inact, inactive sequence.

ubilization of coding sequences (3%) and of DNA (5%) is observed.

Micrococcal nuclease digestion has been shown previously to induce rearrangement of histone H1 (Caron & Thomas, 1981; Lawson & Cole, 1982), which might cause a specific precipitation of transcribed sequences. To excluded this possibility, we prepared and digested nuclei in 5 mM KCl, which prohibits a migration of H1 (Thomas & Rees, 1983), rather than in 60 mM KCl plus 40 mM NaCl. Nearly all of the transcribed sequence i partitions with the insoluble fraction from nuclei digested in the presence of 5 mM KCl. We also determined the H1 content in fraction P2 from nuclei digested at 100 mM monovalent cations relative to that in undigested nuclei as 0.6 (mean of four determinations). Thus, rearranged H1 does not seem to specifically precipitate transcribed sequences.

The partitioning of transcribed sequences with fraction P2 is in contrast to the fractionation of the flanking sequences. Sequences extending on the 5′ side over about 10 kb (sequences b–f), designated as class I flanking sequences, fractionate into fractions S1 and P2 but are significantly depleted in fraction S2. Since coding sequences are depleted in S1 but class I flanking sequences are enriched in S1, the division between these two classes of sequences is clear-cut. The five probes (j–n) used on the 3′ side are complementary to flanking sequences up to 15 kb away from the 3′ end of the gene. The sequences k–n, which are located farthest away from the 3′ end, differ significantly in their partitioning from the 5′ flanking sequences: they are not enriched in any chromatin fraction. Therefore, they are assigned to a separate category designated as class II flanking sequences. The partitioning of sequence j, which is located closer to the 3′ end of the gene, is related to that of class I flanking sequences on the 5′ side, as it shows a slight depletion in fraction S2. We, therefore, tentatively assign this sequence as class I flanking. However, the partitioning of sequence j does differ drastically from that of sequence k next to it on its 3′ side. A similar case is sequence a, the partitioning of which resembles that of the neighboring class I flanking sequence b, although its enrichment in fraction S1 is not very drastic. It seems that the division between class I and class II flanking sequences is not clear-cut but is rather characterized by a gradual change in partitioning. However, the assignment of sequences a and j as class I flanking sequences is supported by a disturbed nucleosomal repeat pattern obtained from these sequences in response to micrococcal nuclease digestion. A disturbed repeat, possessing only diffuse mono- and dinucleosomal bands, is a specific feature of class I flanking sequences; class II flanking sequences have a normal nucleosomal periodicity (see below). The globin genes are not expressed in hen oviduct and, thus, a unique *Eco*RI–*Hin*dIII fragment from the adult β-globin gene was used as a prototype to study the partitioning of inactive sequences during chromatin fractionation (Wood et al., 1981; Villeponteau et al., 1982). Figure 2 shows that this sequence partitions with fractions S2 and P2 but is significantly depleted in fraction S1. Thus, the low-salt chromatin fractionation procedure employed defines four categories of sequences within the lysozyme gene domain: (i) Transcribed coding sequences fractionate into the insoluble fraction P2. (ii) Eleven kilobases of flanking sequences on the 5′ side and probably about 4 kb on the 3′ side (class I flanking sequences) partition with fractions S1 and P2. (iii) Class II flanking sequences (sequences located distal of class I flanking sequences) are not enriched in any chromatin fraction. (iv) The inactive β^A-globin gene partitions with fractions S2 and P2. This differential partitioning is highly reproducible and was observed in at least 20 independent experiments.

Digestion with DNase I and micrococcal nuclease has been used repeatedly to establish that transcribed genes and a defined length of flanking sequences are more sensitive to nuclease digestion than inactive genes [for review, see Igo-Kemenes et al. (1982)]. The ovalbumin gene, for example, lies in a 100-kb DNase I sensitive domain harboring this and two other genes (Lawson et al., 1982). Fractionation of the transcribed coding sequences into an insoluble fraction seems to be in conflict with the preferential nuclease sensitivity of active genes. We, therefore, examined the nuclease sensitivity of a representative sequence from each of the four categories of sequences defined by the fractionation procedure. In practice, oviduct nuclei were digested with micrococcal nuclease to three extents, and four aliquots of the purified DNA of each sample were electrophoresed and transferred onto different nitrocellulose filters. Each filter was then hybridized with a ^{32}P-labeled probe of sequences c, i, and n and of the β^A-globin gene sequence, respectively.

Following digestion with 20 and 30 units of micrococcal nuclease, bulk DNA exhibits a typical nucleosomal ladder (Figure 3, ethidium), which by linear regression analysis possesses an average repeat length of 197 ± 4 bp, in agreement with a value reported by Compton et al. (1976). After Southern blot hybridization, a nucleosomal ladder, which is even more extended than that of bulk DNA, is also observed along the β^A-globin sequence (Figure 3); yet, the repeat length of the β^A-globin ladder is significantly greater than that of bulk DNA (212 ± 4 bp). Similar results are obtained by monitoring the response of class II flanking sequence n to nuclease digestion; an extended nucleosomal ladder possessing a repeat length of 195 ± 5 bp is generated. The rate of digestion of the globin sequence and of sequence n is comparable to that of bulk DNA. However, the coding sequence i is processed into a continuum of fragment lengths and is digested at a significantly faster rate than bulk DNA, as visualized most easily after digestion with 5 units of micrococcal nuclease (Figure 3, lanes a). Class I flanking sequence c is initially also

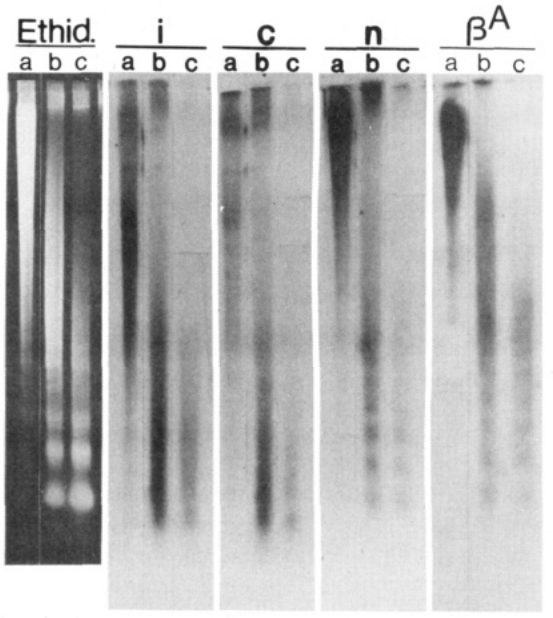

FIGURE 3: Chromatin structure of coding, class I, and class II flanking lysozyme gene sequences and inactive β-globin sequences. Hen oviduct nuclei were digested with micrococcal nuclease (5, 20, and 30 units of enzyme per milliliter, lanes a, b, and c, respectively). Purified DNA samples were electrophoresed 4-fold on 1.5% agarose gels. Gels were stained with ethidium bromide (Ethid.) and photographed. The DNA was then Southern blotted onto nitrocellulose filters and hybridized with the indicated ^{32}P-labeled probes. Complementary sequences were visualized by autoradiography.

processed into a nondiscrete pattern of DNA length and after extensive digestion gives rise to very diffuse mono- and dinucleosomal bands. Identical results were obtained with class I flanking sequences a, b, d, and j (results not shown). The diffuse mono- and dinucleosomal sequence c fragments are 350 ± 7 and 165 ± 6 bp, respectively (corresponding dinucleosomal fragments of bulk DNA, β^A-globin sequence, and class II flanking sequence n are ≈ 390, ≈410, and ≈380 bp, respectively). Thus, coding, class I flanking, and class II flanking sequences are organized into distinct chromatin structures with respect to the appearance of a nucleosome repeat and to the rate of nuclease digestion. Coding sequences lack a nucleosome repeat, class I flanking sequences possess a disturbed nucleosome repeat organization, and class II flanking sequences generate an extended regular nucleosomal ladder. Coding and class I flanking sequences are cleaved at a significantly faster rate than class II flanking sequences and the inactive globin sequence.

The results of the dot-blot hybridization experiment (Figure 2) show that specifically the coding sequences from hen oviduct chromatin partition nearly exclusively with fraction P2. Sequence f, which maps very closely to the coding sequences from −550 to −161 (Renkawitz et al., 1984), partitions differently (with fractions S2 and P2). This suggests that partitioning of coding sequences with the insoluble chromatin fraction is related to transcription. To test this hypothesis, we used hen liver, where the lysozyme gene is not expressed, to examine the partitioning of coding and class I flanking sequences during chromatin fractionation. Hen liver nuclei lack the Ca/Mg-dependent endonuclease present in some mammalian species (Hewish & Burgoyne, 1973; Strätling et al., 1984), and thus, they were also digested with micrococcal nuclease. Following fractionation it is found that sequences i and c partition with fractions S2 and P2 and that both sequences are nearly lacking in fraction S1 (Figure 4). Thus, the partitioning of inactive lysozyme gene sequences in liver chromatin is identical with

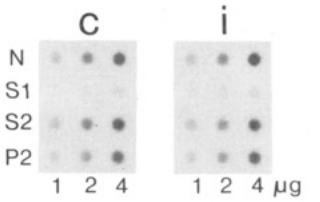

FIGURE 4: Partitioning of inactive lysozyme gene chromatin from hen liver. Hen liver nuclei were digested with micrococcal nuclease, and the purified DNA from chromatin fractions S1, S2, and P2 was dot-blotted onto nitrocellulose filters. The relative abundances of class I flanking sequence c and coding sequence i were measured by hybridization with the respective ^{32}P-labeled probes.

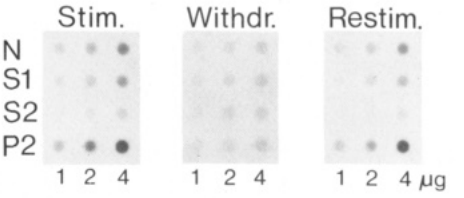

FIGURE 5: Effect of transcriptional cessation on the partitioning of lysozyme gene coding sequences. Oviduct nuclei from primary stimulated, withdrawn, and restimulated immature chicks were digested with micrococcal nuclease, and chromatin fractions S1, S2, and P2 were prepared. The relative abundance of coding sequence i was then determined by dot-blot hybridization.

that of the inactive β^A-globin sequence in oviduct chromatin (see Figure 2).

In order to further support the above suggestion that the partitioning of coding lysozyme gene sequences from hen oviduct nuclei with the insoluble chromatin fraction is related to transcriptional activity, we used immature chicks in which differentiation of the oviduct and transcription of the egg white protein genes have been precautiously induced by estrogen application (Kohler et al., 1969). When these chicks are withdrawn from the hormonal stimulus, transcription of the egg white protein genes is shut off (Hynes et al., 1977). By reapplication of steroid hormones transcription is rapidly reinitiated. Figure 5 shows that, following fractionation of oviduct chromatin from primary stimulated chicks, the coding sequence i partitions with fraction P2, very similar to the partitioning of this sequence in oviduct chromatin from laying hens (see Figure 2A). In response to hormone withdrawal, sequence i is not significantly enriched in any chromatin fraction, resembling the partitioning of class II flanking sequences in the laying hen oviduct (compare to Figure 2A). After secondary stimulation of the oviduct, partitioning of sequence i with fraction P2 is restored. Thus, the partitioning of coding sequences with low-salt insoluble nuclear material is specifically observed when the lysozyme gene is transcribed.

Discussion

Our results show that fractionation of oviduct chromatin by a low-salt procedure defines three classes of sequences within the chicken lysozyme gene domain. Transcribed coding sequences partition with low-salt insoluble material (fraction P2). About 11 kb of flanking sequences on the 5′ side and probably about 4 kb on the 3′ side (class I flanking sequences) partition with fractions S1 and P2; fraction S1 is released from oviduct nuclei during nuclease digestion. Class II flanking sequences comprise sequences located distal of class I flanking sequences; they are not enriched in any chromatin fraction. Finally, a fourth class of sequences is represented by the inactive adult β-globin gene, which partitions with fractions S2 and P2. Thus, the low-salt fractionation procedure has the potential to define and partially separate four functionally different classes of sequences. The classification defined by

the chromatin fractionation is supported by analysis of the rate of digestion and of the nucleosome repeat in response to micrococcal nuclease digestion. Coding sequences lack a canonical nucleosome repeat, class I flanking sequences possess a disturbed nucleosome repeat, and class II flanking sequences and the inactive β^A-globin sequence generate an extended nucleosomal ladder. Coding and class I flanking sequences are more readily digested by micrococcal nuclease than class II flanking sequences and the β^A-globin sequence. Thus, the surprising situation arises that the lysozyme gene, comprising 3.8 kb, is flanked by a severalfold larger region (approximately 15 kb) of DNA packed into a related chromatin structure. The relatively large size of the flanking region with elevated nuclease sensitivity may be related to the diversity with which the lysozyme gene is regulated in different tissues. In the oviduct the gene is under steroid hormonal control, while it is constitutively expressed in chicken macrophages (Fritton et al., 1984). Within the class I flanking region Fritton et al. (1983) previously mapped eight DNase I hypersensitive sites. These sites most likely possess regulatory significance, as they are diversely expressed in different tissues and under different hormonal states (Fritton et al., 1984).

The enrichment of transcribed coding sequences in fraction P2 is close to the maximum possible enrichment for a sequence, and very little (about 10%) of these sequences partition with fractions S1 and S2. Tubular gland cells, which synthesize the lysozyme, represent about 90% of the total oviduct cells (Harris et al., 1975). Thus, about 10% of lysozyme gene coding sequences in oviduct chromatin are expected to partition like the inactive β^A-globin sequence (i.e., with fractions S2 and P2). Control experiments show that the specific fractionation of transcribed coding sequences into the low-salt insoluble fraction P2 does not result from a rearrangement of H1. First, we obtained the same results with nuclei prepared and digested at 5 mM KCl, which minimizes H1 rearrangement (Thomas & Rees, 1983). Second, fraction P2 is slightly depleted of histone H1. Further, the differential fractionation of the four categories of sequences cannot be explained on the basis of a simple size fractionation. The Southern hybridization experiment shows that coding sequences, which partition with the low-salt insoluble fraction, are most readily digested with micrococcal nuclease. The inactive globin gene, the majority of which ($\approx$75%) is solubilized with Tris–EDTA–EGTA, is much less cleaved under the same conditions. Thus, the partitioning of the most heavily cleaved sequences with fraction P2 is in opposition to what one would have expected from DNA in an insoluble fraction. We cannot exclude the possibility that transcribed coding sequences associated with an insoluble nuclear structure during the experimental procedure. If chromatin simply aggregates or attaches to an insoluble chromatin structure, it does so in relationship to the transcription of coding sequences. The class I flanking sequences, which are enriched in the soluble fraction S1, fall within the region where the DNase I hypersensitive sites upstream and downstream of the lysozyme gene have been mapped (Fritton et al., 1983). However, cutting at these sites contributes only marginally to the enrichment of class I flanking sequences in fraction S1, since digestion with micrococcal nuclease was much stronger than the extent of digestion needed to detect hypersensitive sites. The boundary between coding and class I flanking sequences appears to be very sharp (at least on the 5′ side), since the 3′ end of sequence f, which partitions significantly differently from the coding sequences, maps very closely to the start site of transcription (at −161). On the other hand, the boundary between class I flanking and class II flanking sequences is rather diffuse, since there are only small differences between the partitioning of the farthest upstream and downstream class I flanking sequences, respectively, and that of the neighboring class II flanking sequences.

Our results are closely related to those obtained by use of a 2 M NaCl procedure (Berezney & Coffey, 1975). This procedure yields a chromatin fraction, called matrix, that has been shown repeatedly to be enriched in actively transcribed sequences (Jackson et al., 1981, 1982; Robinson et al., 1982, 1983; Ciejeck et al., 1983; Hentzen et al., 1984). Among the many reports the studies by O'Malley and co-workers on the X–Y–ovalbumin gene cluster are most relevant to our work (Ciejek et al., 1983). They showed that selectively transcribed coding sequences fractionate into the nuclear matrix but not sequences upstream or downstream of the gene cluster. We obtained very similar results by using the low-salt fractionation procedure and by performing a more detailed analysis on the lysozyme gene. This has to be regarded as valuable additional information and not merely repetition, since the 2 M NaCl procedure has been questioned for its significance in determining chromatin structure (Kirov et al., 1984). A low-salt fractionation procedure has also been applied in a study on the chromatin structure of immunoglobulin light chain genes (Rose & Garrard, 1984). Transcribed κ light chain gene sequences were found to be enriched in fractions analogous to our S1 and P2 fractions. The authors further noticed a similar partitioning of transcriptionally inactive allelic κ light chain gene sequences and potentially active λ light chain gene sequences. The latter observation, however, is in contrast to our work, which shows a close correlation between the association of lysozyme gene coding sequences with an insoluble nuclear structure and the transcriptional event along these sequences. Ryoji & Worcel (1985) reported that plasmid DNA injected into germinal vesicles of *Xenopus* oocytes fractionates into two types of minichromosomes: insoluble and soluble. The insoluble minichromosomes are more sensitive to digestion and lack a nucleosomal periodicity. Thus, these minichromosomes are similar in structure to the transcribed coding sequences of the lysozyme gene. Bellard et al. (1977) and Bloom & Anderson (1978, 1979) have shown that mononucleosome-length DNA fragments from micrococcal nuclease digested chicken oviduct nuclei are severalfold enriched in the ovalbumin gene when these fragments are separated from the purified DNA. These observations are consistent with our results in Figure 3 showing that transcribed lysozyme gene sequences in laying hen oviduct nuclei are preferentially digested by micrococcal nuclease. Bloom & Anderson (1978) also reported that a mononucleosome-size nucleoprotein fraction released from nuclei during digestion is enriched in ovalbumin gene sequences. One explanation for the apparent variance with our results and those of Rose and Garrard and of Ryoji and Worcel is that different extents of digestion have been used. Rose & Garrard (1984) observed that the distribution of active κ light chain gene sequences among chromatin fractions is dependent on the extent of nuclease digestion. At low levels of digestion, the majority of the active sequences resides in the insoluble chromatin fraction, and upon continued digestion, this material is processed into the soluble chromatin fractions and becomes 6-fold enriched in fraction S1. It is possible that Bloom and Anderson used an extent of digestion that produced such a processing of active sequences from the insoluble chromatin fraction to chromatin fraction S1.

The differential partitioning of flanking sequences suggested that the specific fractionation of lysozyme gene coding se-

quences from hen oviduct chromatin is related to the active transcription of the gene. This suggestion is supported by our results of the partitioning experiment using hen liver chromatin. Lysozyme gene coding and class I flanking sequences partition with fractions S2 and P2, very similar to that for the β^A-globin sequence in oviduct chromatin. The above suggestion is supported further by the partitioning experiment of coding sequences during primary stimulation, withdrawal, and secondary stimulation of the oviduct in immature chicks. The partitioning of nontranscribed coding sequences during fractionation of oviduct chromatin from withdrawn chicks resembles that of class II flanking sequences and is, therefore, in contrast to that of transcribed coding sequences. Thus, only transcribed coding sequences show a specific and selective partitioning with an insoluble chromatin fraction.

The structural basis for the specific partitioning of transcribed sequences with an insoluble chromatin fraction is not known. Our results only indicate that either a component of the transcriptional complex or a constituent, or constituents, specific for transcribed chromatin mediates this partitioning. Since micrococcal nuclease cleaves certainly at multiple sites within the coding sequences, this indicates further that there are multiple sites of association with the insoluble nuclear structure. This conclusion is related to the matrix association of ovalbumin gene sequences, of which the major portion maps in the nontranscribed flanking regions and of which only a very small portion is transcribed (Ciejek et al., 1983; Robinson et al., 1983). Other investigators (Mirkovitch et al., 1984) used a strong ionic detergent (lithium diiodosalicylate) to obtain an insoluble nuclear fraction. A rather short sequence in the nontranscribed spacer region of the histone gene cluster of *Drosophila melanogaster* fractionated into this substructure. Our low-salt method and the procedure using lithium diiodosalicylate are certainly not comparable, since the detergent removes histones and many nonhistone proteins while our conditions are more gentle.

ACKNOWLEDGMENTS

We thank C. Grade and M. Hallupp for competent technical assistance, Dr. J. Nowock for the gift of diethylstilbesterol-filled silicone tubes, Dr. R. Renkawitz for probe f, and Dr. D. Engel for the chicken β-globin clone.

Registry No. Lysozyme, 9001-63-2.

REFERENCES

Baldacci, P., Royal, A., Bregegere, F., Abastado, J. P., Carni, B., Daniel, F., & Kourilsky, P. (1981) *Nucleic Acids Res. 9*, 3575–3588.

Beermann, W. (1952) *Chromosoma 5*, 139–198.

Bellard, M., Gannon, F., & Chambon, P. (1977) *Cold Spring Harbor Symp. Quant. Biol. 42*, 779–791.

Berezney, R., & Coffey, D. S. (1975) *Science (Washington, D.C.) 189*, 291–293.

Bloom, K. S., & Anderson, J. N. (1978) *Cell (Cambridge, Mass.) 15*, 141–150.

Bloom, K. S., & Anderson, J. N. (1979) *J. Biol. Chem. 254*, 10532–10539.

Britten, R. J., Graham, D. E., & Neufeld, B. R. (1974) *Methods Enzymol. 29*, 363–418.

Caron, F., & Thomas, J. O. (1981) *J. Mol. Biol. 146*, 513–537.

Ciejek, E. M., Tsai, M.-J., & O'Malley, B. W. (1983) *Nature (London) 306*, 607–609.

Compton, J. L., Bellard, M., & Chambon, P. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 4382–4386.

Denhardt, D. (1966) *Biochem. Biophys. Res. Commun. 23*, 641–646.

Dolan, M., Sugarman, B. J., Dodgson, J. B., & Engel, J. D. (1981) *Cell (Cambridge, Mass.) 24*, 669–677.

Dolan, M., Dodgson, J. B., & Engel, J. D. (1983) *J. Biol. Chem. 258*, 3983–3990.

Elgin, S. C. R. (1981) *Cell (Cambridge, Mass.) 27*, 413–415.

Foe, V. E., Wilkinson, L. E., & Laird, C. D. (1976) *Cell (Cambridge, Mass.) 9*, 131–146.

Fritton, H. P., Sippel, A. E., & Igo-Kemenes, T. (1983) *Nucleic Acids Res. 11*, 3467–3485.

Fritton, H. P., Igo-Kemenes, T., Nowock, J., Stretch-Jurk, U., Theisen, M., & Sippel, A. E. (1984) *Nature (London) 311*, 163–165.

Harris, S. E., Rosen, J. M., Means, A. R., & O'Malley, B. W. (1975) *Biochemistry 14*, 2072–2081.

Hentzen, P. C., Rho, J. H., & Bekhor, I. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 304–307.

Hewish, D. R., & Burgoyne, L. A. (1973) *Biochem. Biophys. Res. Commun. 52*, 504–510.

Hynes, N. E., Groner, B., Sippel, A. E., Nguyen-Huu, M. C., & Schütz, G. (1977) *Cell (Cambridge, Mass.) 11*, 923–932.

Hynes, N. E., Groner, B., Sippel, A. E., Jeep, S., Wurtz, T., Nguyen-Huu, M. C., Giesecke, K., & Schütz, G. (1979) *Biochemistry 18*, 616–624.

Igo-Kemenes, T., Hörz, W., & Zachau, H. G. (1982) *Annu. Rev. Biochem. 51*, 89–121.

Jackson, D. A., McCready, S. J., & Cook, P. R. (1981) *Nature (London) 292*, 552–555.

Jackson, D. A., Caton, A. J., McCready, S. J., & Cook, P. R. (1982) *Nature (London) 296*, 366–368.

Jost, J.-P., & Seldran, M. (1984) *EMBO J. 3*, 2005–2008.

Kafatos, F. C., Jones, C. W., & Efstratiadis, A. (1979) *Nucleic Acids Res. 7*, 1541–1552.

Kirov, N., Djondjurov, L., & Tsanev, R. (1984) *J. Mol. Biol. 180*, 601–614.

Kohler, P. O., Grimley, P. M., & O'Malley, B. W. (1969) *J. Cell Biol. 40*, 8–27.

Lawson, G. M., & Cole, R. D. (1982) *J. Biol. Chem. 257*, 6576–6580.

Lawson, G. M., Knoll, B. J., March, C. J., Woo, S. L. C., Tsai, M.-J., & O'Malley, B. W. (1982) *J. Biol. Chem. 257*, 1501–1507.

Lindenmaier, W., Nguyen-Huu, M. C., Lurz, R., Stratmann, M., Blin, N., Wurtz, T., Hauser, H. J., Giesecke, K., Land, H., Jeep, S., Grez, M., Sippel, A. E., & Schütz, G. (1979) *Proc. Natl. Acad. Sci. U.S.A 76*, 6196–6200.

Mirkovitch, J., Mirault, M.-E., & Laemmli, U. K. (1984) *Cell (Cambridge, Mass.) 39*, 223–232.

Nowock, J., & Sippel, A. E. (1982) *Cell (Cambridge, Mass.) 30*, 607–615.

Renkawitz, R., Schütz, G., von der Ahe, D., & Beato, M. (1984) *Cell (Cambridge, Mass.) 37*, 503–510.

Rigby, P. W. J., Dieckmann, M., Rhodes, C., & Berg, P. (1977) *J. Mol. Biol. 113*, 237–251.

Robinson, S. I., Nelkin, B. D., & Vogelstein, B. (1982) *Cell (Cambridge, Mass.) 28*, 99–106.

Robinson, S. I., Small, D., Idzerda, R., McKnight, G. S., & Vogelstein, B. (1983) *Nucleic Acids Res. 11*, 5113–5130.

Rocha, E., Davie, J. R., van Holde, K. E., & Weintraub, H. (1984) *J. Biol. Chem. 259*, 8558–8563.

Rose, S. M., & Garrard, W. T. (1984) *J. Biol. Chem. 259*, 8534–8544.

Ryoji, M., & Worcel, A. (1985) *Cell (Cambridge, Mass.) 40*, 923–932.

Sanders, M. M. (1978) *J. Cell Biol. 79*, 97–109.

Southern, E. (1975) *J. Mol. Biol. 98*, 503–517.

Strätling, W. H., & Klingholz, R. (1981) *Biochemistry 20*, 1386-1392.
Strätling, W. H., Grade, C., & Hörz, W. (1984) *J. Biol. Chem. 259*, 5893-5898.
Thomas, J. O., & Rees, C. (1983) *Eur. J. Biochem. 134*, 109-115.
Villeponteau, B., Landes, G. M., Pankratz, J. M., & Martinson, H. G. (1982) *J. Biol. Chem. 257*, 11015-11023.
Weintraub, H., & Groudine, M. (1976) *Science (Washington, D.C.) 193*, 848-856.
Wood, W. L., Nickol, J., & Felsenfeld, G. (1981) *J. Biol. Chem. 256*, 1502-1505.

Secondary Structure of Halorhodopsin†

B. K. Jap* and S.-H. Kong
Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720
Received June 25, 1985

ABSTRACT: Ultraviolet circular dichroism (CD) spectroscopy in the interval from 190 to 240 nm has been used to estimate the secondary structural content of halorhodopsin (hR), a light-driven chloride pump isolated from the membranes of *Halobacterium halobium*. Least-squares curve fitting of the CD spectrum for hR solubilized with octyl glucoside yields an α-helical content of $\sim$50% and a β-structure content of $\sim$30%. The CD spectrum of hR is unaffected by the absence or presence of chloride ions or by the ionic strength of the medium. The CD spectrum of halorhodopsin is very similar to that of bacteriorhodopsin, indicating that these light-driven pumps possess nearly identical fractions of α- and β-secondary structures.

Halorhodopsin (hR) is one of three retinal-containing proteins found in *Halobacterium halobium*. The biological function of hR is to serve as a light-driven, electrogenic chloride pump, transporting chloride ions inward across the membrane (Schobert & Lanyi, 1982). Very little is known about the precise physiological role of hR, about its structure, or about the molecular mechanism by which chloride is translocated across the membrane.

Halorhodopsin has many similarities and dissimilarities when compared to bacteriorhodopsin (bR). Both proteins contain a retinal moiety that is apparently in a similar environment as suggested by resonance Raman spectroscopy (Smith et al., 1984; Alshuth et al., 1985), indicating that structures surrounding the retinal in both of these proteins are very similar. On sodium dodecyl sulfate (SDS) gel electrophoresis, hR shows a slightly greater mobility than that of bR, suggesting that hR has a similar molecular mass (i.e., 26 000 daltons). The amino acid compositions of hR and bR are quite similar when residues are grouped as being hydrophobic, neutral, and polar (Ariki & Lanyi, 1984; Sugiyama & Mukohata, 1984). Proteolytic fragments of hR and bR are also similar when the proteins are partially digested with *Staphylococcus aureus* V8 protease (Hegemann et al., 1982). However, hR also shows distinct spectroscopic and photochemical properties. For example, in contrast to the case of bR, the photocycle intermediates and kinetics, as well as the position of hR absorption maximum, are affected by the presence of chloride ions (Ogurusu et al., 1982; Stoeckenius & Bogomolni, 1982; Lanyi & Schobert, 1983; Steiner et al., 1984). There is evidence that hR has a distinct chloride binding site (Schobert et al., 1983; Falke et al., 1984) involving a sulfhydryl group (Ariki & Lanyi, 1984). Furthermore, hR and bR lack immunological cross-reactivity (Hegemann et al., 1982), suggesting a significant difference in their primary sequences.

It was not known whether there is any similarity between the secondary structure of bR and hR. In this paper, we show from ultraviolet CD spectroscopy that these two proteins possess a similar secondary structural content.

Materials and Methods

The procedures for growth of *Halobacterium halobium* (JW-12 and R_1) and for isolation of purple membrane are those described by Oesterhelt & Stoeckenius (1974). The purification of hR follows the method described by Taylor et al. (1983). To ensure that a highly purified protein was obtained, isolated hR was rerun on a small hydroxylapatite column as described by Taylor et al. (1983). The purity of the isolated hR was determined by SDS gel electrophoresis. Fractions with no visible impurity as observed on SDS gels were collected and dialyzed in the dark against several changes of 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes)/3 M NaCl buffer (pH 7) at 4 °C. After removal of octyl D-β-glucopyranoside (Calbiochem) by dialysis, hR was dialyzed at 4 °C in the dark against two changes of 1 L of 20 mM sodium phosphate buffer (pH 7). The dialyzed hR was resolubilized for 6 h in 20 mM sodium phosphate, pH 7, containing 15 mM octyl D-β-glucopyranoside (octyl glucoside) and then immediately used for spectral studies.

The basic method used for solubilization of purple membrane follows that described by Dencher & Heyn (1978). Five milligrams of purple membrane in 20 mM sodium phosphate buffer (pH 6.9) was centrifuged at 50000*g* for 40 min. The pellet was resuspended in 20 mL of 20 mM phosphate buffer, pH 6.9, containing 1.3% octyl glucoside. The suspension was kept in the dark at room temperature ($\approx$22 °C) for 30 h and then centrifuged at 150000*g* (averaged value) for 30 min. The pellet, if any, was discarded.

Circular dichroism was measured with a Jasco J-500A spectropolarimeter. The dichrograph was calibrated with a

† This work was supported by the Director, Office of Energy Research, Office of Health and Environmental Research of the U.S. Department of Energy, under Contract DE-AC03-76SF00098 and by National Institutes of Health Research Grant GM23325.

0006-2960/86/0425-0502$01.50/0